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# **Effects on Brain Development of Prenatal Inhibition of Kynurenine-3-Monooxygenase**

In the partial fulfilment of the requirements for the degree of Doctor of  
Philosophy.

**Institute of Neuroscience and Psychology**

**College of Medical, Veterinary and Life Science**

***University of Glasgow***

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BSc (Hons)

## Abstract

Much is known about the disease pathology related to schizophrenia, however, little is known with regards to its aetiology. Recent evidences suggest a neurodevelopmental hypothesis for schizophrenia where environmental factors including: infection, stress and malnutrition, can adversely affect the pregnant mother thereby elevating the risk for schizophrenia developing in the offspring during adulthood (Meyer *et al.*, 2008d; Meyer and Feldon, 2009; 2012; Forrest *et al.*, 2012; Meyer, 2013). Since a variety of viral and bacterial infections in animal models have demonstrated to increase the risk in schizophrenia, it is proposed that factors common to the immune response may mediate this link. While many laboratories have reported several behavioural abnormalities following maternal immune activation, we sought to examine molecular changes following poly(I:C) exposure, a synthetic viral mimetic, in the pregnant mother and assessed a range of protein markers with known developmental roles, since an appreciable understanding of the molecular alterations taking place would permit suitable therapies to follow. Interestingly, poly(I:C) was able to induce a range of changes resembling those observed during schizophrenia, where the major NMDA receptor subunit GluN1 and  $\alpha$ -Synuclein was reduced in postnatal day 21 animals born to mothers treated with poly(I:C) during gestation days 14, 16 and 18. Furthermore, these changes suggest a mechanism by which maternal immune activation may lead to the subsequent emergence of schizophrenia.

Another aspect of this work examined the role of the kynurenine pathway on brain development. There is increasing evidence suggesting the involvement of the kynurenine pathway, a biochemical pathway responsible for the oxidative metabolism of tryptophan, in the disease pathology of schizophrenia, including neurodegenerative disorders such as Parkinson's, Alzheimer's and Huntington's disease (Giorgini *et al.*, 2005; Ting *et al.*, 2009; Bonda *et al.*, 2010). Since immune activation induces the activation of the kynurenine pathway, it was hypothesised that alterations in central kynurenine concentrations during development may be involved in mediating the subsequent increased risk for schizophrenia (Forrest *et al.*, 2013, Khalil *et al.*, 2013, 2014). As very little is known about the physiological activity of the kynurenine pathway during development, we sought to examine the potential consequence of disrupting this pathway and examining its effects upon brain development. Therefore, a kynurenine monooxygenase inhibitor, Ro61-8048, was administered to pregnant rats during gestation day 14, 16, and 18, that would inhibit the synthesis of the neurotoxic metabolite quinolinic acid, while redirecting the pathway to increase the neuroprotectant

kynurenic acid. Brain development was assessed by examining changes in protein expression of markers intimately involved in synaptic transmitter release machinery, neurogenesis and many aspects of neuronal development. Interestingly, we found the kynurenine pathway is highly active during brain development, and induces a variety of changes in protein markers that may be involved in precipitating a range of neuronal and cognitive deficits. While Ro61-8048 induced no changes in the embryo brains at 5 and 24 h following treatment, delayed changes were seen in postnatal day 21 animals displaying a decrease in RhoB expression as examined in the western blots. Since the full blown symptoms of schizophrenia become apparent during early adulthood, we sought to examine any changes in protein expression in postnatal day 60 animals in regions of the cortex, hippocampus, midbrain and cerebellum. Interestingly, profound alterations were seen in doublecortin and the netrin receptors responsible for axonal guidance. Perhaps the most striking protein change in the postnatal day 60 animals is the significant alteration induced in the expression of disrupted in schizophrenia (DISC)-1, a protein strongly linked with schizophrenia. Glutamate function was assessed as indicated by the density of glutamate transporters, VGLUT-1 and VGLUT-2, in the CA1 region of the hippocampus of postnatal day 60 animals using immunocytochemistry. While the relative density of glutamate transporters were substantially increased, there were no changes in the GABA transporters, indicating that while GABA transmission remained the same, glutamate function may have increased in the absence of an increase in synaptic connections. Spine densities of pyramidal neurons in the hippocampus were also examined, using the golgi-impregnation method, to reveal a significant loss in spines of the apical and basal dendrites, consistent with reports in schizophrenia. To conclude, the kynurenine pathway is highly active during development, and alterations in central kynurenines during pregnancy, as induced by environmental factors such as stress and infection, may be involved in the subsequent emergence of neurodevelopmental disorders.

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## Authors Declaration

I declare that the work presented in this thesis is entirely of my own with other contributions being clearly stated as work performed by other in a parallel study.

Signature.....

Omari S. Khalil

## List of Publications

The research presented within this thesis has been published, either in part or in full, contributing to the following publications:

Forrest, C.M., **Khalil, O.S.**, Pizar, M., Smith, R.A., Darlington, L.G., & Stone, T.W. 2012. Prenatal activation of Toll-like receptors-3 by administration of the viral mimetic poly(I:C) changes synaptic proteins, N-methyl-D-aspartate receptors and neurogenesis markers in offspring. *Molecular Brain*, **5**, 22

Forrest, C.M., **\*Khalil, O.S.**, Pizar, M., Darlington, L.G., & Stone, T.W. 2013. Prenatal inhibition of the tryptophan–kynurenine pathway alters synaptic plasticity and protein expression in the rat hippocampus. *Brain Research*, **1504**, 1-15

**\*Khalil, O.S.**, Forrest, C.M., Pizar, M., Smith, R.A., Darlington, L.G., & Stone, T.W. 2013. Prenatal activation of maternal TLR3 receptors by viral-mimetic poly(I:C) modifies GluN2B expression in embryos and sonic hedgehog in offspring in the absence of kynurenine pathway activation. *Immunopharmacology and Immunotoxicology*, **35(5)**, 581-593

Forrest, C.M., **Khalil, O.S.**, Pizar, M., Darlington, L.G., & Stone, T.W. 2013. Changes in synaptic transmission and protein expression in the brains of adult offspring after prenatal inhibition of the kynurenine pathway. *Neuroscience*, **254C**, 241-259

**\*Khalil, O.S.**, Pizar, M., Forrest, C.M., Vincenten, M.C.J., Darlington, L.G., & Stone, T.W. 2014. Prenatal inhibition of the kynurenine pathway leads to structural changes in the hippocampus of the adult rat offspring. *European Journal of Neuroscience*. In Press.

\* co-first author papers

## Abbreviations

$\alpha$ -Syn	$\alpha$ -synuclein
1-MT	1-Methyl-Tryptophan
3-HK	3-Hydroxykynurenine
5-HIAA	5-Hydroxyindoleacetic Acid
5-HT	5-Hydroxytryptamine
5-HT <sub>2C</sub>	5-Hydroxytryptamine-2C Receptor
5-HTT	Serotonin Transporter
6-OHDA	6-hydroxydopamine
Ach	Acetylcholine
AD	Alzheimer's Disease
AIDS	Human Immunodeficiency Virus
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	Blood-Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BPB	Blood-Placental Barrier
CA1	Cornu Ammonis 3
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
COX	Cyclo-oxygenase
COX-2	Cyclo-oxygenase-2
CSF	Cerebro Spinal Fluid
D <sub>2</sub> R	Dopamine 2 Receptor
DA	Dopamine
DAT	Dopamine Transporters
DCC	Deleted in Colorectal Cancer
DISC-1	Disrupted in Schizophrenia-1
DOPAC	dihydroxyphenylacetic acid
EGF	Epidermal Growth Factor
FTIs	Farnesyltransferase Inhibitors
GABA	Gamma-Aminobutyric Acid
GPCR	G-Protein Coupled Receptor
GPCR-35	G-Protein Coupled Receptor-35
HD	Huntington's Disease
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus-1
HPA axis	Hypothalamic-Pituitary-Adrenal axis
Htt	Huntington Gene
HVA	homovanillic acid
IBGC	Idiopathic Basal Ganglia Calcification
IDO	Indoleamine-2,3-Dioxygenase
IFN- $\alpha$	Interferon- $\alpha$
IFN- $\beta$	Interferon- $\beta$
IFN- $\gamma$	Interferon- $\gamma$
IL-1	Interleukin-1
IL-1 $\beta$	Interleukin-1 $\beta$
IL-1ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-10	Interleukin-10
KA	Kainic Acid

KAT	Kynurenine Aminotransferase
KAT 1	Kynurenine Aminotransferase 1
KAT 2	Kynurenine Aminotransferase 2
KMO	Kynurenine-3-Monooxygenase
KYN	Kynurenine
KYNA	Kynurenic Acid
L-DOPA	Levodopa
LGP	lateral global pallidus
LI	Latent Inhibition
LPS	Lipopolysaccharide
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MK-801	Dizocilpine
MPTP	1-methyl 4-phenyl-2,3,6-tetrahydropyridine
NAc	Nucleus Accumbens
NfκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NO	Nitric Oxide
NMDA	N-methyl-D-aspartate
NRG-1	Neureglin-1
P	Postnatal day
PCP	Phencyclidine
PD	Parkinson's Disease
PGE2	Prostaglandin E2
PGs	Prostaglanins
PNS	Peripheral Nervous System
Poly(I:C)	Polyriboinosinic-polyribocytidilic acid
PPI	Prepulse Inhibition
PSD-95	Post Synaptic Density-95
QUIN	Quinolinic Acid
RhoA-GG	RhoA-Geranylgeranylated
RhoB-F	RhoB-Farnesyled
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
Ro61-8048	3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulphonamide
ROCK	Rho-associated kinase
ROS	Reactive Oxygen Species
SNARE	Soluble NSF Attachment Protein Receptor
SynPhy	Synaptophysin
SynT	Synaptotagmin
TDO	Tryptophan-2,3-Dioxygenase
TH	Tyrosine Hydroxylase
TLR-2	Toll-like Receptor-2
TLR-4	Toll-like Receptor-4
TLR-3	Toll-like Receptor-3
TGF-α	Tumor Growth Factor-α
TNF-α	Tumor Necrosis Factor-α
TURP	Turpentine
VAMP-1	Vesicle Associated Membrane Protein-1 (Synaptobrevin)
VAMP-2	Vesicle Associated Membrane Protein-2
VGAT	Vesicular GABA Transporter
VGLUT-1	Vesicular Glutamate Transporter-1
VGLUT-2	Vesicular Glutamate Transporter-2



### 1.1 Normal Foetal Development

The correct neurodevelopment of the central nervous system (CNS) and the normal physiological processes of neurogenesis in the developing foetus is imperative for the normal functioning of mammals. The health and well-being of the offspring critically depends upon newly born neurons developing axons that navigate through a complex environment to find their target, while dendrites undergo extensive branching after which functional synapses are established between neuronal populations throughout the developing cortex. This is achieved by efficient neuronal differentiation and migration responding to precise axonal guidance cues, leading to the formation of functional synapses on dendritic spines (Linseman and Loucks, 2008), thereby allowing for accurate synaptic communication to occur between neurons. Although early embryonic development and neurogenesis occurs within the protective environment of the maternal placenta, maternal pregnancy (gestation) is a critically vulnerable period for the developing embryo. Early foetal damage during gestation can prove severely debilitating later in life displaying several permanent neurochemical, cyto-architectural and behavioural abnormalities in the offspring.

### 1.2 Abnormal Foetal Development

Embryonic development is known to be severely affected by a variety of factors including: maternal smoking, alcohol exposure and drug use. Maternal smoking has been reported to affect foetal growth, thereby reducing the birth weight of the offspring (Lobel *et al.*, 2008), and is implicated with behavioural problems in childhood and adolescence such as ADHD, aggression and depression (Ashford *et al.*, 2008; Indredavik *et al.*, 2007). Maternal alcohol exposure also increases the risk of behavioural problems, cognitive deficits and stress reactivity in the developing offspring (Sayal *et al.*, 2007; Haley *et al.*, 2006), while prenatal exposure to drugs like cocaine and marijuana are characterised by heightened risks for behavioural problems and cognitive deficits in neonatal animals (Bennett *et al.*, 2008; Goldschmidt *et al.*, 2008). As maternal smoking, alcohol exposure and drug use are voluntary acts; their detrimental effects on the developing foetus can largely be avoided. However, maternal infection is now also a recognised environmental risk factor implicated in the aetiologies of several disorders, including schizophrenia. Since maternal infection is largely independent upon individual lifestyles, novel pharmacological treatments or vaccinations may prove fruitful in the endeavour to minimise the risk of early foetal damage.

### 1.2.1 Maternal Influenza Exposure Increases the Risk for Schizophrenia

Schizophrenia is a mental disorder affecting around 1% of the population characterised by deficits in cognitive abilities like abnormal and incoherent thought processes, flattening of the emotional responses, lack of motivation often resulting in social withdrawal, and the onset of auditory and visual hallucinations. A strong genetic tendency is observed in familial studies (Harrison and Weinberger, 2005) where twin studies of schizophrenic patients suggest concordance rates of 45% for monozygotic twins and 14% for dizygotic twins (Sullivan *et al.*, 2003). The aetiology of schizophrenia remains unknown, however, recent evidence is increasingly beginning to view schizophrenia as a neurodevelopmental disorder caused by disruptions in early brain development.

In 1988 an intriguing paper was published which reported a striking 50% increase in the risk for schizophrenia in Finnish individuals whose mothers were exposed to the 1957 A2 influenza virus, during the second trimester of pregnancy (Mednick *et al.*, 1988). However, their findings, based upon case records obtained from psychiatric hospitals, failed to confirm whether the mothers of the schizophrenic patients had indeed contracted the influenza A viral infection during the epidemic. Strikingly, 9 out of 15 studies have replicated these findings demonstrating a positive correlation with the influenza virus and the subsequent increased risk for schizophrenia (O'Callaghan *et al.*, 1991; Adams *et al.*, 1993). Furthermore, an increase in schizophrenic births in late winter and spring also indicates a potential influenza infection (Machon *et al.*, 1983). Mednick later reported obstetric records showing 13 of 15 pregnant mothers in their second trimester of pregnancy during the influenza epidemic were recorded to display symptoms of influenza viral infection (Mednick *et al.*, 1994). Since the first report, several studies have examined these claims using various sources including: health records, hospital records, maternal recall and birth cohort studies showing 3 out of 5 birth cohort reports attest to this correlation (Wright *et al.*, 1995; Byrne *et al.*, 2007), while the majority of approximately 50 studies undertaken indicate a 5-15% excess for schizophrenic births in the northern hemisphere during the months of January and March (Boyd *et al.*, 1986).

More conclusively, recent studies have examined archived maternal serum thereby providing serologic evidence to confirm the positive association between maternal exposure to the influenza virus followed by the subsequent increase in risk of schizophrenic offspring (Mortensen *et al.*, 2007; Brown *et al.*, 2004, 2005; Buka *et al.*, 2008). Furthermore, studies revealed that patients exposed to the influenza infection *in-utero* were accompanied by executive dysfunction (Brown *et al.*, 2009) and significantly reduced intelligence rates (Eriksen *et al.*, 2009), findings consistent with schizophrenia as being recognised to be accompanied by

cognitive deficits and executive dysfunction (Rusch *et al.*, 2007). These studies provide convincing evidence demonstrating maternal exposure to the influenza virus heightens the risk for the subsequent development of schizophrenia during adulthood.

On the contrary, other groups have shown an association between prenatal exposure to the influenza virus and mental retardation (Takei *et al.*, 1995), while another reported an increased risk in major affective disorder following maternal influenza exposure in the second trimester of pregnancy (Machon *et al.*, 1997). Additionally, maternal infection has also been reported to increase the risk of autism and cerebral palsy developing in the offspring later in life. Together, this demonstrates the effects of prenatal infection with the influenza virus are not specific for or restricted to schizophrenia, but is also a risk factor associated with a wide range of CNS disorders.

### *1.2.2 Immune Activation Precipitates Developmental Disorders*

Since influenza is a common seasonal virus affecting the respiratory system and its detrimental association for the increased risk of schizophrenia and related disorders is concerning, Canada and America advised pregnant women to be vaccinated against influenza (Boksa, 2008). However, many studies with other viruses and bacterial infections have replicated the findings of influenza infection. Currently, there is compelling evidence for an increased susceptibility for schizophrenia and related disorders following prenatal exposure to infection with a variety of viral pathogens including: influenza (Mednick *et al.*, 1988), rubella (Brown *et al.*, 2000), toxoplasma gondii (Brown *et al.*, 2005; Mortensen *et al.*, 2007), measles, polio, herpes simplex (Buka *et al.*, 2001; 2008) including infection with bacterial pathogens and genital and/or reproductive infections (Meyer and Feldon, 2009). While these several studies provide convincing evidence for a rationale suggesting prenatal infection with bacterial or viral entities contributes to the emergence of the disease, they all fall short in proving causation. Furthermore, reflecting on the diversity of infectious agents involved, it is hypothesised that factors common to the immune system may provide the critical link between maternal infection and the increased risk for schizophrenia (Patterson, 2002). Promising studies demonstrate pro-inflammatory cytokines to be a principal key player in mediating this link (Bell *et al.*, 2004), leading to the hypothesis that induction of the maternal immune response may be important in altering early brain development thereby increasing the risk of schizophrenia and related disorders.

### 1.2.3 Neuroinflammation and Parkinson's Disease

Many disorders are known to be accompanied by neuroinflammation including Parkinson's disease (PD) and Alzheimer's disease (AD). In contrast to schizophrenia, Parkinson's disease is a neurodegenerative disorder primarily affecting elderly people aged over 65, and is associated with the progressive loss of catecholergic neurons in the substantia nigra (SN) with a reduction in dopamine (DA) and acetylcholine (ACh) transmitters. While much is known about the disease pathology, very little is known with regards to the aetiology of Parkinson's disease. Neuroinflammation is recognised as an integral part of its pathophysiology, however, it is unknown whether this is the driving force in neurodegeneration or whether it simply represents a response to neuronal death. While neuroinflammation has long been considered secondary to PD, involved in the self-perpetuating deleterious events that lead to protracted neuronal degeneration, recent evidence suggests that members of the immune system may also be able to initiate CNS disorders like PD. Indeed, case-control studies showed PD is significantly related to influenza and herpes simplex complex infections (Vlajinac *et al.*, 2013). Another study provided evidence of an association between CNS infections and a higher future risk of PD (Fang *et al.*, 2012), while one group showed the presence of influenza A virus inside neuromelanin granules as well as in tissue macrophages within the substantia nigra from post-mortem PD brains (Rohn *et al.*, 2011). While these studies suggest some infectious diseases may play a role in the development of PD, other independent groups have shown that members of the immune system can indeed recapitulate some Parkinsonian-like symptoms. This is supported by a study showing encephalitis or viral infection with the H5N1 influenza strain in mice could cause Parkinsonism by inducing neurodegeneration of neurons in the substantia nigra (Jang *et al.*, 2009). Furthermore, overexpression of interferon-gamma (IFN- $\gamma$ ) in mice resulted in age-progressive nigrostriatal degeneration and basal ganglia calcification, which is reminiscent of human idiopathic basal ganglia calcification (IBGC ; Chakrabarty *et al.*, 2011), and characterised by neurological features such as Parkinsonism, dystonia, tremor and cognitive decline (Manyam *et al.*, 2001; Baba *et al.*, 2005). Since poly(I:C) is a potent inducer of the interferons, including IFN- $\gamma$ , these studies suggest viral infection may be involved in the initiation of Parkinson's disease. Furthermore, they lend mutual support that maternal immune activation may potentially also be a risk factor for the subsequent early onset of PD during adulthood and may account for, at least in part, the increased incidence of PD following the encephalitis lethargica during 1917-1928.

### 1.3 Immunological Contributors: Cytokines

Immune activation, by either infection or stress, resulting in the rapid induction of cytokines, molecules existing as low-molecular weight proteins produced and secreted by immune competent cells, respond to a number of environmental stimuli mediating a defence against a variety of infectious agents (Patterson, 2002). Receptors activated by the binding of cytokines on target cells initiate intracellular signalling cascades which regulate gene expression within target cells. The role of cytokines varies considerably in activating lymphocytes and in regulating immune cell differentiation and homeostasis, while some cytokines are capable of inducing cell apoptosis and inhibiting protein synthesis. Members of the cytokine family are separated into two main groups according to their principal function. Pro-inflammatory cytokines are involved in the body's early defence against infection where they are responsible for the initiation and progression of inflammation. Examples of pro-inflammatory cytokines include interleukin (IL)-1 $\beta$ , IL-6 and tumour necrosis factor (TNF)- $\alpha$  (Bell *et al.*, 2004; Brown *et al.*, 2009). Conversely, anti-inflammatory cytokines are responsible for limiting the production of pro-inflammatory cytokines thus reducing the degree of inflammation. Therefore, anti-inflammatory cytokines including: IL-10 and transforming growth factor (TGF)- $\alpha$ , are intimately involved in maintaining a state of immunological homeostasis (Meyer *et al.*, 2008a). Since activation of the maternal immune response during pregnancy interferes with the development of the foetus, a phenomenon strongly linked to an increase in subsequent risk of developmental disorders during adulthood, this raises the possibility of novel anti-inflammatory therapeutics to treat such conditions where current anti-psychotic medication has failed.

#### 1.3.1 Cytokine Specific Actions and Dependencies

In an attempt to elucidate the effects of cytokines in mediating developmental damage, the roles of cytokines *in vitro* have been investigated. Studies have shown that out of various cytokines, IL-1 $\beta$  is most capable in proliferating rat mesencephalic progenitor cells into cells with a dopaminergic phenotype (Ling *et al.*, 1998; Potter *et al.*, 1999) while IL-6 is highly efficacious in decreasing the survival of foetal brain serotonin neurons (Jaraskog *et al.*, 1997). Also, IL-1 $\beta$  and IL-6 at low to medium concentrations are equally capable in negatively regulating the survival of foetal midbrain dopaminergic neurons, while at higher concentrations they are able to promote the survival of these cells (Jaraskog *et al.*, 1997). Furthermore, low concentrations of TNF- $\alpha$  disrupts the dendritic development of cortical neurons, while cortical dendritic development can also be disrupted by higher concentrations of IL-1 $\beta$ , IL-6 including TNF- $\alpha$ . This demonstrates that cytokine specific reactions occur

often with a dependency upon their relative concentration in regulating neuronal populations principally affected in schizophrenia.

However, not all effects of specific cytokine reactions appear to be solely dependent upon their relative concentration, since studies have shown the effects of developing cells towards cytokines vary as foetal brain development progresses. Although TNF- $\alpha$  is neurotrophic in early foetal development towards dopaminergic ventral mesencephalic neurons, during later stages of foetal brain development it precipitates neurotoxic effects to the same neuronal population (Doherty, 2007). *In vitro* studies also highlight this altered sensitivity toward signalling cues where embryonic cells cultured as progenitor neurospheres show a greater sensitivity and responsiveness in proliferating in response to basic Fibroblast Growth Factor (bFGF) then to Epidermal Growth Factor (EGF), while postnatal and adult progenitor cells show enhanced proliferation in response to EGF than bFGF (Zhu *et al.*, 1999). This demonstrates that immune activation during different stages of brain development can exert varying effects due to the altered sensitivity or responsiveness of developing cells towards a variety of signalling cues.

This concept has been taken further in a series of experiments to show maternal immune activation in mice exposed to poly(I:C) during different developmental windows precipitates different patterns of subsequent dysfunctions in the brain and behaviour of the offspring. While some brain and behavioural dysfunctions were preserved between the developmental insults occurring during both early/middle or late gestation including: impairments in spatial working memory and the enhanced sensitivity to amphetamine and dizocilpine (Meyer *et al.*, 2007), suggesting a conserved pool of behavioural abnormalities, there also exists a select pool of behavioural dysfunctions that are restricted to developmental damage occurring only during early/middle gestation including: deficiencies in latent and prepulse inhibition (Meyer *et al.*, 2006a; Meyer *et al.*, 2007), and reduced exploratory behaviour (Meyer *et al.*, 2006b). Although these effects may be due to the altered sensitivity and responsiveness of the foetal brain towards the cytokine-specific reactions as development progresses, another mechanism accounting for this may be due to cytokine interference with specific spatiotemporal events occurring at distinct times of neurodevelopment thereby affecting select systems in the foetal brain.

### 1.3.2 Candidates for Acute Cytokine Mediators

Promising studies have emerged associating the relative role of key individual cytokines with the emergence of behavioural deficits *in vivo*. Interestingly, prenatal

administration of exogenous IL-6 mimics the long-term consequences of poly(I:C) treatment in mice producing deficits in prepulse inhibition (PPI) and latent inhibition (LI) in the offspring, while co-administration with IL-6 blocking antibodies with poly(I:C) failed to induce behavioural deficits in latent and prepulse inhibition (PPI), social interaction and exploratory behaviour in the offspring (Smith *et al.*, 2007). Furthermore, prenatal exposure to poly(I:C) in IL-6 knockout mice did not induce deficits in PPI, exploratory behaviour and social interaction. The emerging role of IL-6 has been extended to a rat model of prenatal infection, where IL-6 administered in early gestation (E8, E10 and E12) including late gestation (E16, E18 and E20) produced deficits in spatial learning, neuronal loss and changes in the hippocampus (Samuelsson *et al.*, 2006). A recent study also confirmed these findings where prenatal exposure to poly(I:C) in DISC-1 mutant mice were more sensitive to the effects of maternal immune activation, an observation attributed to substantially elevated levels of IL-6 found in foetal brains. Consistently, co-administration of anti-IL-6 antibodies with poly(I:C) reversed the schizophrenia-related behavioural phenotype in the DISC-1 mutant mice (Lipina *et al.*, 2013).

Similarly, co-administration of IL-1 receptor antagonist (IL-1ra) with LPS in pregnant rats prevents the increase in pro-inflammatory cytokine levels, microglial activation and motor deficits in the offspring (Girard *et al.*, 2010). However, prenatal exposure to IL-1 $\beta$  alone is not sufficient to induce similar behavioural deficits in the offspring during adulthood. Prenatal exposure to IFN- $\gamma$  or TNF- $\alpha$  alone was also incapable of inducing behavioural deficits in the offspring, while poly(I:C) co-administered with soluble IL-1 $\beta$  or IFN- $\gamma$  receptor antagonist during pregnancy failed to prevent behavioural deficits induced by poly(I:C) (Smith *et al.*, 2007). Another group showed inhibiting prostaglandin synthesis with an adequate dose of ibuprofen, a cyclooxygenase (COX) inhibitor, prior to LPS exposure, was insufficient in blocking the reduced neurogenesis observed in P14 animals in response to prenatal exposure with LPS alone (Cui *et al.*, 2009). These studies provide some of the most convincing evidence that IL-6 and IL-1ra may indeed represent key cytokines by which maternal immune activation can induce significant impairments in brain and behaviour, while lending mutual support to the theory that therapies aiming to regulate IL-6 and IL-1ra during prenatal infection may provide an alternative therapy for the treatment of developmental disorders.

Since pro-inflammatory cytokines like IL-6 appear to be leading candidates in mediating considerable brain and behavioural damage following maternal immune activation, the question arises whether enhancing the expression of anti-inflammatory cytokines are equally as efficacious in alleviating these behavioural deficits. In order to address this, some



models of prenatal infection have exposed poly(I:C) to pregnant transgenic mice constitutively overexpressing the anti-inflammatory cytokine IL-10 in macrophages. Interestingly, normal control animals exposed to poly(I:C) were accompanied by behavioural deficits including abnormalities in PPI, LI and exploratory behaviour, while these effects were prevented in animals overexpressing IL-10 that were exposed to poly(I:C) (Meyer *et al.*, 2008a). However, it was also noted that animals overexpressing IL-10, in the absence of prenatal poly(I:C) exposure, resulted in similar deficits in exploratory behaviour and latent inhibition. This suggests IL-10 may have a significant role in providing protection against pro-inflammatory cytokines in the context of prenatal infection only.

#### 1.4 Interplay of a Heterogeneous Aetiology for Schizophrenia

Although maternal infection is associated with an increased incidence in the offspring developing schizophrenia, some studies have failed to show this trait (Morgan *et al.*, 1997; Selten *et al.*, 1999), demonstrating maternal infection on its own is not sufficient to account for such an association. Prenatal exposure to influenza B virus resulted in reduced cognitive performances in children aged 7 and developed schizophrenia in adulthood, whereas no effect was seen in control children who displayed no signs of psychiatric morbidity in adulthood (Ellman *et al.*, 2009). Another study found that maternal exposure to urinary and bladder infections, the risk of schizophrenia was only evident in those individuals who possessed a family history of psychiatric disorders (Clark *et al.*, 2009). It is therefore likely other factors, including genetic and environmental contributions, influence the enhanced risk for schizophrenia, and that maternal infection may incur a heightened risk in individuals already possessing pre-existing vulnerabilities.

It is possible there exist certain gene polymorphisms within individuals making them more vulnerable to the effects of infection. Indeed, recent advances have identified multiple susceptibility genes known to be adversely affected in the foetal brain during prenatal infection, however, the precise nature of environment-gene interactions leading to vulnerabilities in developing schizophrenia remains largely unknown. It is possible these genes may confer susceptibility to particular disorders or confound how the developing embryo may react to maternal infection or adverse environments. In contrast to rodent models of prenatal infection, the majority of cases of maternal infection during human pregnancies are not accompanied by the subsequent development of schizophrenia when the offspring reach adulthood. This clearly demonstrates that firstly, maternal infection does not prove causation of the disorder, and secondly, should maternal infection be involved in the aetiology of



schizophrenia, it may likely serve to interact with genetically determined factors, like genes, may confer a particular vulnerability towards the disorder.

Finally, although genes may predispose an individual to a particular disorder, studies have consistently demonstrated the necessity for continued environmental stressors and conflict to play an integral role in the development of schizophrenia (Dean and Murray, 2005). A variety of environmental factors are now known to increase the risk for schizophrenia including: family conflict, paternal criminality, low social class, inconsistent parenting styles, maternal psychopathology and exposure to acute or chronic stressors (Opler and Susser, 2005). It is therefore believed genetic and environmental factors together have a dynamic interplay leading to the subsequent development of schizophrenia.

### 1.5 Experimental Models of Maternal Infection

Several animal models have been developed in order to investigate the effects of maternal infection, and to address whether prenatal immune activation can cause relative or absolute, transient or more prolonged long-term alterations in CNS function, while providing an invaluable tool to study what these mechanisms may be. These experimental models exist almost exclusively in rodents, and rely on pharmacological agents capable of activating the cytokine response thereby mimicking an infection. These cytokine-activating agents are administered to the pregnant rodent at various doses to induce a strong or weak immune response, ranging from the repeated administration of immunogenic agents throughout pregnancy to a single administration in early or late gestation.

Currently, most research has focused on three principal rodent models of maternal infection, with each model dependent upon the specific pharmacological agent employed. This includes the influenza virus itself, lipopolysaccharide (LPS), and polyriboinosinic-polyribocytidilic acid (poly(I:C)). The use of LPS and poly(I:C) in animal models of prenatal infection over live pathogens, like the influenza virus, is of benefit since these agents illicit the immune response but provide more control over the time course of infection and dosage, allowing for a more regulated approach to identify the precise gestational windows of vulnerability. Consequently, these agents are merely immunogenic stimulants activating their specific toll-like receptors, and since no live bacterial or viral entity exists within drug preparations, these agents fail to activate the full inflammatory response while also remaining incapable of reproducing the entire time course of a live propagating infection.

### 1.5.1 Lipopolysaccharide (LPS)

LPS is a bacterial endotoxin, from Gram-negative bacteria, known to activate toll-like receptor (TLR)-2 and TLR-4 (Triantafilou and Triantafilou, 2002). Systemic administration of LPS activates the innate immune response typically observed following infection with Gram-negative bacteria characterised by cytokine induction, inflammation, fever onset, activation of the hypothalamic-pituitary-adrenal axis and sickness behaviour. LPS bound to TLR-4 on macrophages, and other immune competent cells, initiate a signal transduction cascade activating transcription factors NF $\kappa$ B with the subsequent transcription of genes coding for both pro- and anti-inflammatory mediators including: cytokines, chemokines and proteins of the complement system. LPS induces the pro-inflammatory cytokines: IL-1, IL-6 and TNF- $\alpha$  (Ashdown *et al.*, 2006). Locally produced IL-1 and TNF- $\alpha$  activates fibroblasts and endothelial cells to induce their own synthesis of cytokines, while circulating levels of IL-6 in the brain induces the inducible enzyme COX-2 isoform which is involved in the synthesis of prostaglandins (PGs). This COX-2-induced activation of prostaglandins mediates the rise in core body temperature frequently seen during fevers, a mechanism involving PGE<sub>2</sub> acting on thermo-responsive neurons in the preoptic area of the anterior hypothalamus (Roth *et al.*, 2009).

### 1.5.2 polyriboinosinic-polyribocytidilic acid (Poly(I:C))

Poly(I:C), a synthetic analogue of virus-specific double stranded RNA, is often used as an experimental tool to mimic the acute phase of viral infection. Viruses, as with poly(I:C), activate TLR-3 (Takeuchi and Akira, 2007) and involves the translocation of NF $\kappa$ B to the cell nucleus to induce the expression of pro-inflammatory cytokines: IL-1, IL-6 and TNF- $\alpha$ . Poly(I:C) is also a potent inducer of the type I interferons, INF- $\alpha$  and IFN- $\beta$ , thus mimicking the acute phase response to viral infection (Cunningham *et al.*, 2007). Furthermore, poly(I:C) induces a pro-inflammatory viral response in the absence of an actual viral entity.

Intriguingly, schizophrenia exhibits a unique characteristic maturational delay in disease onset where the full blown spectrum of symptoms only emerges after post-pubertal maturity until early adulthood (Weinberger, 1987). Similarly, this maturational delay is mirrored by the long term effects of prenatal poly(I:C) exposure in rodents since the complete spectrum of poly(I:C)-induced behavioural, cognitive and pharmacological abnormalities only emerge after the post-pubertal stage of development (Rapoport *et al.*, 2005). Although prenatal infection has also been linked to autism spectrum disorders and mental retardation developing in the offspring during adulthood, these are not dependent upon post-pubertal maturation processes which appear to be a distinctly relevant characteristic unique to

schizophrenia, suggesting maternal immune activation may be a more suited model to study the aetiopathological and pathophysiological processes associated with schizophrenia.

#### 1.5.2.1 Reason for Selecting poly(I:C) over LPS

For the purpose of this research, rodent models of prenatal infection were employed while poly(I:C) was used as the immunogenic agent over LPS, since most infections during pregnancy appear to be viral in nature as opposed to bacterial, and secondly, due to issues with the reproducibility of LPS arising from batch-to-batch variations. Since LPS is extracted and purified from the cell wall of bacteria, each batch of LPS usually varies in its pyrogenic and cytokinogenic activity (Rey *et al.*, 1991). It has been demonstrated that equal doses of different batches of LPS may differ in their time course of cytokine induction with the propensity to produce a hypothermic response as opposed to a hyperthermic response (Akarsu and Mamuk, 2007). On the contrary, it is unknown whether different batches of poly(I:C) are accompanied with variations in its immunogenic activity. Finally, since the mothers of postnatal day 21 animals were injected on three separate occasions with poly(I:C), there exists a tolerance to repeated LPS exposure where successive dosing results in attenuated cytokine and fever responses (Chen *et al.*, 2005), whereas this does not occur with poly(I:C).

#### *1.5.3 Timing of Infection and Window of Maximal Foetal Damage*

Initial epidemiological reports implicated viral infection during pregnancy to be associated with an increased prevalence of schizophrenia emerging in the offspring only when the pregnant mother was exposed to the virus in the second trimester of human pregnancy (Mednick *et al.*, 1988; O'Callaghan *et al.*, 1991; Adams *et al.*, 1993). Recent more quantifiable studies examining serologic samples from pregnant mothers exposed to an infection have revealed interesting findings, firstly challenging the view that the second trimester of pregnancy may be the critical time window which may confer maximal risk for the offspring developing schizophrenia during adulthood, and secondly, highlighting the undue overemphasis on second trimester infections. Serologic evidence demonstrates influenza viral infection during the first trimester of pregnancy is associated with yet the greatest risk of schizophrenia developing in the offspring (Brown *et al.*, 2004). Although a variety of studies have shown rodent models of maternal infection exposed to poly(I:C) during early, middle and late stages of gestation are all efficacious in inducing brain abnormalities, one study examining the effects of poly(I:C) exposure on mice during E9 (early/middle gestation) and E17 (late gestation) indicated prenatal exposure to poly(I:C) during early/middle gestation (E9) may precipitate a more extensive impact on brain and behavioural deficits relevant to schizophrenia (Meyer *et al.*, 2007). Furthermore, since early/middle pregnancy (E9) in a

mouse roughly corresponds to the middle/end of the first trimester of human pregnancy and late pregnancy (E17) in a mouse roughly corresponds to the middle trimester of human pregnancy, this experimental mouse model of prenatal infection lends further support to the theory that the first trimester, as opposed to the second trimester, of human pregnancy may indeed represent the time period where prenatal immune activation confers maximal vulnerability to foetal brain development. Indeed, during late gestation many physiological systems are already established and an immunological insult with LPS or poly(I:C) may result in a marginal disruption on brain development, while interference during a stage where these processes are largely still underway with neurogenesis and synaptogenesis still occurring, an immunogenic insult at this stage would induce maximal disruption in the normal development of these systems thereby inducing significant impairments in brain function while further maturational processes would build upon and incorporate earlier immune-compromised neuronal networks. However, since infections in the second trimester of pregnancy are also efficacious (albeit to a lesser extent) at inducing brain abnormalities, models of maternal infection corresponding to both the first and second trimesters of human pregnancy are of relevance in understanding the pathophysiological alterations occurring in schizophrenia.

### 1.6 Effects of Anti-psychotic Drugs in Immune Challenged Offspring

Since prenatal exposure to infectious agents is known to elevate the risk of schizophrenia, with rodents exhibiting symptoms reminiscent of schizophrenia during adulthood, considerable interest has peaked into assessing whether current anti-psychotic drugs are able to treat, or marginally correct, behavioural deficits in adult offspring exposed to LPS or poly(I:C) prenatally. Research groups have employed two principal treatment schedules. Firstly, to assess the effectiveness of acute and moderate doses, adult rodent offspring were treated acutely with anti-psychotics prior to behavioural testing. Studies utilising this treatment schedule have shown a single injection of haloperidol, a typical anti-psychotic, was efficacious in correcting deficits in PPI in mice exposed to the influenza virus prenatally (Shi *et al.*, 2003). These findings have also been observed in mice following acute treatment with other anti-psychotics including chlorpromazine and clozapine. The results have been extended to rats exposed prenatally to LPS and poly(I:C) where acute treatment with anti-psychotics reversed changes in PPI (Romero *et al.*, 2007) and LI (Zuckerman and Weiner, 2005). This demonstrates that both mice and rats are responsive to even acute treatment with anti-psychotic medication during adulthood and lends further support to the theory that offspring born to LPS or poly(IC)-treated animals during pregnancy indeed display

a schizophrenia-like behavioural phenotype that respond to current anti-psychotic drug treatment.

A second treatment schedule, employed in these animal studies, attempts to replicate the time scale required for anti-psychotic efficacy in humans, and therefore involved adult offspring being treated with anti-psychotics for several weeks prior to behavioural testing. Studies utilising this treatment schedule demonstrated the repeated treatment with clozapine over a period of two weeks was able to correct deficits in novel object recognition tests in mice exposed to poly(I:C) during gestation (Ozawa *et al.*, 2006). A larger study on mice prenatally exposed to poly(I:C) were treated with either haloperidol, clozapine or the SSRI anti-depressant fluoxetine over a period of four weeks from P35-P65 while behavioural testing was done during adulthood at P90-P120. Their results demonstrated selective responses where different drugs were able to correct selective abnormalities. Clozapine corrected deficits in PPI and LI, haloperidol corrected deficits in latent inhibition including the increased AMPA and MK-801-induced locomotion, while fluoxetine corrected PPI and the increased AMPA-induced locomotion (Meyer *et al.*, 2008a, 2008b, 2008c). The mechanisms by which these drugs are capable of correcting abnormalities during adulthood caused by prenatal immune activation remain largely elusive, since anti-psychotics often have multiple actions on multiple receptor systems in addition to antagonising dopamine D2 receptors, all which may have contributing effects. Although current anti-psychotics are effective in treating the symptoms of schizophrenia in these animals, their practical application in humans remain unsatisfactory, while it is increasingly becoming recognised that these drugs may indeed be responsible for the reduction in cortical and hippocampal volume previously attributed to disease progression (Lieberman *et al.*, 2005; Moncrieff and Leo, 2010; Cacabelos *et al.*, 2011).

Considering the increasingly evident neurodevelopmental aetiology for schizophrenia where environmental factors such as infection or stress may predispose individuals to an increased susceptibility of developing the disorder, anti-inflammatory therapy for treating inflammatory reactions during early development have been proposed. The significance of this was illustrated in a cohort study showing 63.3% of 4967 pregnant women were exposed to a viral or bacterial infection during pregnancy, while infections were more common during earlier stages of pregnancy than in the three months prior to conception (Collier *et al.*, 2009). However, maternal exposure to analgesics, including anti-inflammatory agents and codeine, in the second trimester of pregnancy is also associated with an increased risk for schizophrenia (Sorensen *et al.*, 2004), while cytokine knockout studies show pregnancies to spontaneously abort, suggesting cytokines and their receptors are constitutively expressed during pregnancy

and are important in the development of the foetus. Indeed, TNF- $\alpha$  at low physiological levels is involved in neuronal survival, while prenatal exposure to poly(I:C) and LPS significantly reduces its expression in the foetal brain (Urakubo *et al.*, 2001; Gilmore *et al.*, 2005), which may contribute towards neuronal loss. Since overexpression of IL-10 in macrophages also precipitates behavioural deficits in the offspring (Meyer *et al.*, 2008a), it becomes apparent that a potential treatment should aim to restore the relative balance between pro- and anti-inflammatory cytokines during maternal immune activation. Moreover, although clinical trials show anti-inflammatory therapy used in conjunction with anti-psychotics to demonstrate a superior treatment schedule in alleviating schizophrenia symptoms when compared with anti-psychotic treatment alone (Meyer *et al.*, 2011), employing the use of broad non-specific anti-inflammatory or immunosuppressive treatments during a long term illness like schizophrenia may increase the incidence of increased sensitivity to infections. Therefore, future therapeutic approaches would likely emerge from deciphering intracellular pathways that underlie the convergence of environmental-immune influences and their effects on neurodevelopment.

#### *1.6.1 The Kynurenine Pathway: A Missing Link to Developmental Disorders?*

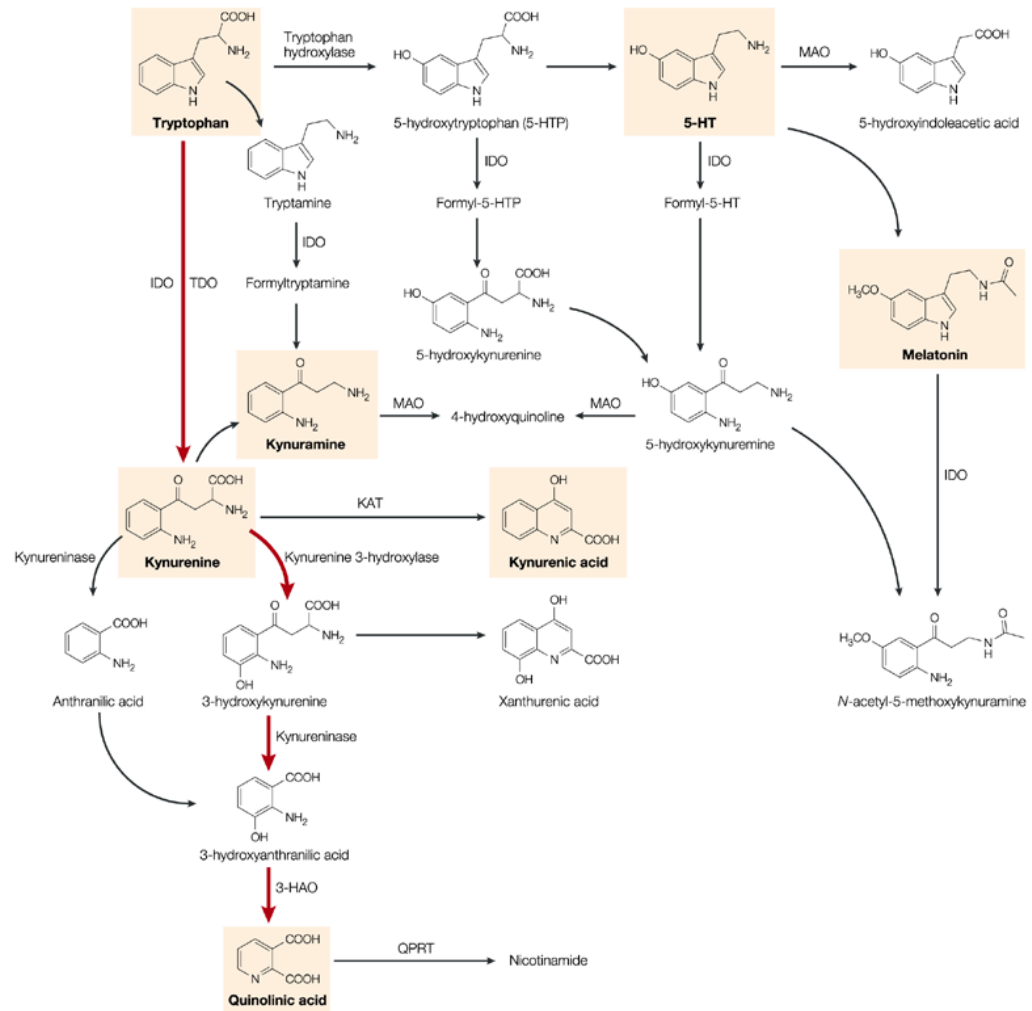
While attempting to restore the cytokine imbalance may prove challenging, this entire field of study to date has neglected the significant contribution and immunomodulatory role of the kynurenine pathway, which is readily activated during neuroinflammation generating a variety of neurotoxic metabolites. It is most probable that many of the effects of maternal immune activation are mediated, at least in part, by the generation of neurotoxic kynurenine metabolites that may be involved in mediating damage previously attributed to cytokines alone, suggesting the kynurenine pathway may indeed mediate this convergent link between environment-immune influences on development. Therefore, since the targeting of the overall immune system appears to be guarded by a complex set of interactions, modifying the activity of the kynurenine pathway to prevent the synthesis of neurotoxic metabolites while simultaneously promoting the synthesis of neuroprotective metabolites serves a valid drug target and an exciting prospect of a kynurenine based therapy to treat disorders involving immune activation. Indeed, the kynurenine pathway as a potential drug target for the treatment of neurodegenerative diseases including: Huntington's, Parkinson's, Alzheimer's and cerebral hyperexcitability disorders including: ischemia, stroke and epilepsy are well under way including some compounds being patented (Muchowski *et al.*, 2011; Zisapel *et al.*, 2012; Andersen *et al.*, 2012) while others have reached clinical trials. However, in order to evaluate the therapeutic use of kynurenine based drugs for treatment of developmental disorders, the consequences of modifying the physiological activity of the kynurenine pathway during

development needs to be addressed, which is the centre of intense research within our laboratory.

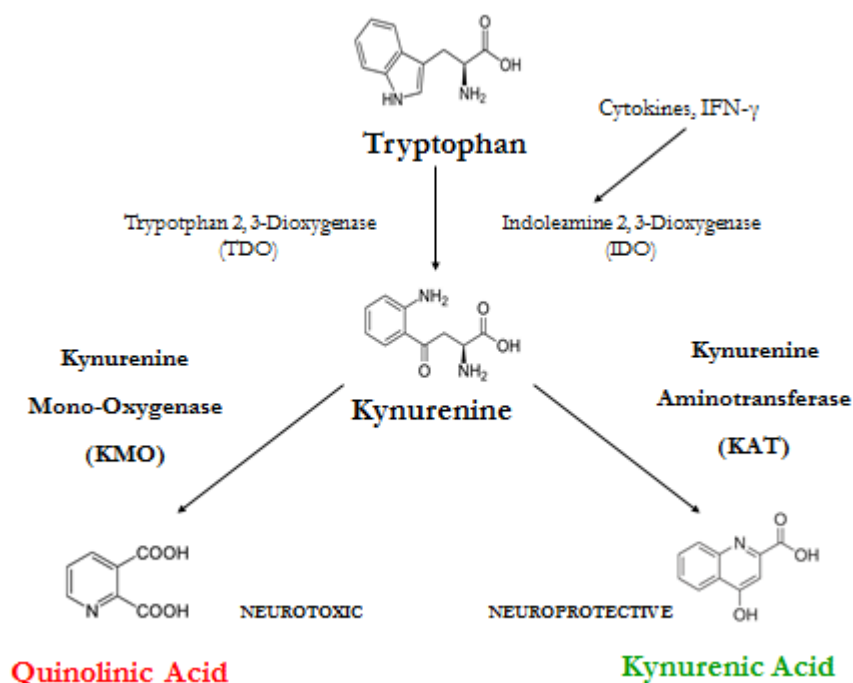
### 1.7 The Kynurenine Pathway

L-tryptophan, an essential amino acid, is a precursor for the synthesis of several biologically active compounds including the neurotransmitter serotonin, the hormone melatonin, and tryptamine. Approximately 99% of endogenous tryptophan not utilised for protein synthesis is metabolised along the kynurenine pathway (figure 1-1), thereby forming the major route of oxidative tryptophan metabolism, generating a variety of neuroactive metabolites. The conversion of tryptophan is initiated by induction of the rate-limiting heme-containing enzymes either tryptophan-2,3-dioxygenase (TDO), an enzyme located in the liver, or more importantly, by the extra-hepatic indoleamine-2,3-dioxygenase (IDO) enzyme which is found in most other tissues. Tryptophan is metabolised by reduction of the heme iron by either superoxide or cytochrome b5 (Maghazal *et al.*, 2008) thereby forming N-formylkynurenine. This intermediary compound is then further metabolised to form kynurenine (KYN), a precursor for two principal divergent arms of the pathway (figure 1-2). The precursor protein kynurenine can be metabolised by two principle enzymes ultimately leading to the formation of either neurotoxic or neuroprotective metabolites. Kynurenine-3-monooxygenase (KMO) is responsible for the conversion of kynurenine (KYN) into quinolinic acid (QUIN), while kynurenine aminotransferase (KAT) converts KYN into kynurenic acid (KYNA) (Schwarcz *et al.*, 2009).





**Figure 1-1. The oxidative metabolism of tryptophan along the kynurenine pathway.** The majority of unused dietary tryptophan is metabolised along the kynurenine pathway producing a variety of neurotoxic metabolites including quinolinic acid (QUIN), 3-hydroxykynurenine (3-HK), and 3-hydroxyanthranilic acid (3-HAA), while kynurenic acid (KYNA) forms the major neuroprotective metabolite of this pathway. Tryptophan not metabolised via the kynurenine pathway is used to produce the transmitter 5-hydroxytryptamine (5-HT) including the hormone melatonin. This diagram has been reproduced with permission from the author Trevor Stone from the University of Glasgow.



**Figure 1-2. Simplified diagram of the kynurenine pathway.**

The oxidative metabolism of tryptophan is initiated by the rate-limiting enzyme indoleamine-2,3-dioxygenase (IDO) which can be induced by a variety of environmental stressors including infection or pro-inflammatory cytokines. Activation of IDO metabolises tryptophan to produce the precursor protein, kynurenine (KYN), which can undergo further metabolism to produce two principal metabolites. While KYN can be metabolised by kynurenine aminotransferase (KAT) to produce the neuroprotective kynurenine, kynurenic acid (KYNA), during an infection the predominant route of metabolism involves KYN conversion into quinolinic acid (QUIN), a neurotoxic metabolite catalysed by the enzyme kynurenine mono-oxygenase (KMO).

Kynurenines were initially believed to be physiologically inactive metabolites of tryptophan until QUIN was found to selectively activate N-methyl-D-aspartate (NMDA) receptors thereby increasing glutaminergic activity (Stone and Perkins, 1981), albeit with weak agonistic activity with an  $IC_{50}$  of 30-100  $\mu$ M (Schwarcz *et al.*, 2009). Since activation of NMDA receptors is associated with the greatest influx of calcium ions into neurons, over other glutamate receptors, it is known to play an integral role in neurotoxicity and neurodegenerative disorders. Owing to the ability of QUIN to activate NMDA receptors, this kynurenine metabolite has been examined to delineate its physiological significance, including its possible interactions during pathological states. QUIN was found to mediate excitotoxicity leading to neuronal death following application of QUIN to neuronal cell cultures *in vitro* (Kim and Choi, 1987) including direct intracerebral administration *in vivo* (Stone *et al.*, 1987), raising the possibility that QUIN may indeed contribute towards neurodegeneration via

activation of NMDA receptors. This was confirmed by the finding that kainic acid (KA) produced axon-sparing lesions of the striatum and neuronal damage which was significantly reduced by antagonists at the NMDA receptor, including by preventing the synthesis of QUIN, suggesting this damage is mediated, at least in part, by the toxic amounts of QUIN which is produced following treatment (Behan and Stone, 2000).

The emerging role of the kynurenine pathway as a regulator of NMDA receptors was strengthened by the finding that kynurenic acid (KYNA) was a neuro-inhibitory compound capable of antagonising all excitatory ionotropic glutamate receptors, including the NMDA receptor via inhibition of the glycine recognition site (Perkins and Stone, 1982), albeit with a low potency of around 7  $\mu$ M. Furthermore, KYNA is also efficacious in inhibiting the  $\alpha$ -7 nicotinic receptor with a similar potency (Hilmas *et al.*, 2001), while recent reports have demonstrated agonistic actions at the aryl hydrocarbon receptor and an orphan G-protein coupled receptor (GPCR)-35 with a low potency of 30-100  $\mu$ M, although their functions remain poorly understood (Wang *et al.*, 2006; DiNatale *et al.*, 2010). Importantly, KYNA has been shown to protect against many of the effects of QUIN via inhibition of its excitotoxic actions by directly inhibiting the excessive activation of glutamate and NMDA receptors. Since elevated levels of QUIN are associated with higher frequencies of epileptiform activity, higher levels of KYNA are effective anticonvulsants and provide protection against QUIN-induced excitotoxic lesions, ischemia and traumatic brain injury (Vamos *et al.*, 2009). Although no clear physiological functions for these kynurenine metabolites have emerged in the brain, there are reports demonstrating the ability of KYNA and QUIN in modulating glutamate and acetylcholine (ACh) receptor function in diseased states (Schwarcz *et al.*, 2009; Costantino, 2009), however, their endogenous contribution towards neurotransmission in normal CNS physiology remains poorly understood. Owing to the principle actions whereby kynurenine metabolites regulate NMDA receptors, the kynurenine pathway has become a novel target for aspiring drugs to modulate glutamate receptor activity in a different way. Although, four isoenzymes for kynurenine aminotransferase (KAT) have been identified in the mammalian brain, only KAT I and KAT II are widely associated with the transamidation of KYN into KYNA (Guidetti *et al.*, 2007; Yu *et al.*, 2006), while the pharmacological inhibition of KAT II decreases KYNA concentrations (Alkondon *et al.*, 2004) consequently increasing NMDA activity and glutamate release, a mechanism predicted to be useful in treatments of glutaminergic and cholinergic hypofunction like schizophrenia and Alzheimer's disease. Furthermore, in diseases involving hyperexcitability and neurodegeneration, including Parkinson's and Huntington's disease, selective KMO inhibitors aimed at reducing the neurotoxin QUIN in favour of KYNA production illustrate promising results.

### 1.7.1 Immune Activation Induces IDO Expression

All component enzymes and metabolites of the kynurenine pathway are present within the PNS and CNS. Peripherally, kynurenine enzymes can be found in the liver (in the case of TDO), endothelial cells, pericytes of the blood-brain barrier (BBB ; Owe-Young *et al.*, 2008) and immune-competent cells of the monocyte and macrophage lineage (Heyes *et al.*, 1992), while centrally, all component enzymes and metabolites are expressed, albeit at much lower levels, in glial cells while little or no expression is found in neurons. However, the precursor protein KYN in the peripheral circulation can be uptaken through the BBB into glial cells, as well as being produced in the brain, thereby increasing central kynurenine levels and their resulting metabolites (Fukui *et al.*, 1991). Therefore, tissue damage from CNS trauma is accompanied by induction of the inflammatory response with activation and invasion of peripheral macrophages into the CNS, including activation of central microglial cells. This neuroinflammation is also accompanied by the activation of the kynurenine pathway, where macrophages and microglial cells produce a variety of cytotoxins in addition to QUIN (Espey *et al.*, 1997).

Indeed, *in vivo* studies using the bacterial endotoxin LPS have shown IDO expression to be increased in excess of 100-fold (Saito *et al.*, 1992), and QUIN immunoreactivity is increased in the brain and lymphoid tissues within 24 hours, primarily within dendritic cells and macrophages (Espey *et al.*, 1995). Infection with HIV in patients with AIDS-dementia complex, a disorder involving a persistent neuroinflammation, is also accompanied with increases in QUIN (Brew *et al.*, 1995), while patients suffering from various neurological dysfunctions, with a neuroinflammatory component, saw significantly elevated concentrations of QUIN in many brain regions including the prefrontal cortex, hippocampus, basal ganglia and cerebellum, when compared with patients whose neurological dysfunction existed in the absence of neuroinflammation (Heyes *et al.*, 1992a). *In vitro* studies have also yielded consistent findings where interferon- $\gamma$  can readily increase IDO, KMO and NOS activity in macrophages and microglia (Alberati-Giani and Cesura, 1998), while tryptophan and KYN is readily converted into QUIN thereby elevating its concentration in human microglia, blood macrophages and human foetal brain cells (Heyes *et al.*, 1992b; Heyes *et al.*, 1996). Infiltrating macrophages following inflammation are also believed to produce 20-30-fold more QUIN than brain microglial cells (Moffet *et al.*, 1997). Another group reported substantial increases in the concentration of QUIN reaching 10.3  $\mu$ M 72 hours post-treatment of human macrophages to INF- $\gamma$ , while co-administration of INF- $\gamma$  with TNF- $\alpha$  produced even higher concentrations of QUIN reaching 16.7  $\mu$ M (Pemberton *et al.*, 1997).

Since basal levels of QUIN in the brain are very low, usually in the range of 0.01  $\mu\text{M}$  and rarely exceeding 1  $\mu\text{M}$  (Stone and Darlington, 2002), it is therefore conceivable and indeed likely that pathological activation of the kynurenine pathway following CNS injury or neuroinflammation may well be efficacious in producing local concentrations of QUIN that are sufficient to substantially activate NMDA receptors to induce neurotoxicity. This is supported by *in vitro* experiments where QUIN at a concentration of 100 nM is sufficient to induce cell death in 40-60% of rat spinal neurons (Giulian *et al.*, 1993), while many reports have shown QUIN concentrations to exist well in excess of neurotoxic levels following exposure to cytokines (Pemberton *et al.*, 1997). However, although true for *in vitro* studies, this has not been adequately addressed in *in vivo* models. These studies illustrate infection or inflammation is accompanied with the induction of IDO and substantially elevated levels of QUIN as opposed to KYNA. The induction of IDO initiating tryptophan metabolism leads to the increased formation of the precursor protein kynurenine (KYN) which is then metabolised either into QUIN or KYNA, however, given that resident and reactive microglia, following CNS injury or neuroinflammation, including activated infiltrating macrophages from the periphery harbour very little KAT activity in favour of KMO enzyme activity, this accounts for the majority conversion of KYN into QUIN (Guillemin *et al.*, 2003), thereby substantially increasing the ratio between QUIN:KYNA to mediate neurotoxicity and neurodegeneration.

#### 1.7.1.1 QUIN-Induced Damage in HIV and AIDS-Dementia Complex

Some of the strongest evidence of kynurenine involvement in CNS disorders comes from the acquired immunodeficiency syndrome (AIDS) accompanied with dementia. It is reported around 20% of patients suffering from AIDS experience significant CNS involvement associated with cognitive decline, motor dysfunction and behavioural abnormalities (Power and Johnson, 1995). Since NMDA receptor activation precipitates brain damage in AIDS patients (Lipton, 1998), there is mounting evidence implicating the neurotoxin kynurenine metabolite QUIN in the development of this CNS dysfunction during AIDS-dementia complex. A mouse model of immunodeficiency syndrome induced by administration of LP-BM5 murine leukaemia virus displayed an increase in QUIN concentration in the blood and brain measured 2 weeks post-infection with a maximal increase seen at 16 weeks post-infection, while a non-pathogenic but equivalent strain of virus induced no change. Interestingly, both the viral load and the concentration of QUIN was significantly reduced following anti-viral treatment, (Nagra *et al.*, 1994; Sei *et al.*, 1996), suggesting the mechanism of anti-virals may also involve the reduction of QUIN thereby improving the symptoms of the disease. Furthermore, infected monkeys show increased

levels of IDO activity (Saito *et al.*, 1991) with heightened levels of QUIN in the CSF (Rausch *et al.*, 1994), with up to a 400-fold increase above basal levels. Consequently, it was also noted the substantial elevation of QUIN correlated directly with the levels of neurological impairment observed in these animals (Rausch *et al.*, 1994; Heyes *et al.*, 1998). Post-mortem analysis of brain tissue from AIDS-patients revealed a 23-fold increase when compared with controls (Achim *et al.*, 1996). Furthermore, the levels of QUIN in the CSF of HIV infected patients are significantly elevated by 20-fold, an increase directly correlating with the degree of CNS dysfunction including deficits in cognition and motor control (Martin *et al.*, 1992), while brain levels itself may exceed those reported in the CSF by a 300-fold increase (Heyes, *et al.*, 1998). Interestingly, zidovudine, an anti-viral drug used in the treatment of HIV, was able to significantly reduce the concentration of QUIN which was accompanied by neurological improvements. Also, IDO expression and activity is significantly increased in brains of patients suffering from AIDS-dementia complex, when compared with AIDS patients in the absence of dementia (Sardar and Reynolds, 1995), confirming the rise in QUIN is indeed from the increased oxidative metabolism of tryptophan along the kynurenine pathway (Huengsberg *et al.*, 1998). Although these studies examined the levels of QUIN in HIV infected adults, these findings have been extended to children with symptomatic HIV-1 infection, albeit with lower levels. One study reported a 4-fold increase in QUIN levels from the CSF of children infected with HIV when compared with controls, which when treated with zidovudine returned to control values (Brouwers *et al.*, 1993). Collectively, these studies lend substantial support that QUIN is an important metabolite in the disease pathology of HIV and AIDS-dementia complex likely involved in mediating central neurodegenerative damage, potentially contributing towards the ensuing neurological dysfunction. This is further strengthened by the reduction of QUIN followed by improvements in neurological function following anti-viral treatment. Furthermore, since the concentration of QUIN is far exceeding neurotoxic levels in these disorders, often over substantial periods of time, it is likely to be a major contributor of towards neurotoxicity. This is consistent with early reports demonstrating micromolar concentrations of QUIN to be toxic after several hours (Galarraga *et al.*, 1990) while even submicromolar concentrations induce neuronal toxicity in cultured experiments if the levels of QUIN are maintained for several weeks (Whetsell and Schwarcz, 1989). Toxicity here is presumably since basal levels of QUIN in the brain are extremely low and not detected suggesting a rapid metabolism, therefore the prolonged exposure of QUIN even at non-toxic doses may induce toxicity. Cell culture experiments of human central neurons exposed to QUIN at a concentration of 350 nM for 5 weeks, reduce cell density and a microtubule-associated protein thereby affecting cellular cyto-structure (Kerr *et al.*, 1998). Since neurotoxic



concentrations of QUIN are achieved in AIDS-patients, these *in vitro* studies emphasise the potential contribution chronically elevated QUIN may exert during AIDS-dementia complex.

#### 1.7.1.2 QUIN-Induced Damage in Huntington's Disease

Kynurenine metabolites are implicated in a large number of diseases including Parkinson's, Alzheimer's, Huntington's and AIDS-dementia (Stoy *et al.*, 2005). It remains unknown whether alterations in kynurenine metabolites precipitate a direct cause to these diseases, or simply exert a secondary role in disease progression thereby prolonging or exacerbating symptoms. However, due to remarkably striking similarities identified between the neurotoxic effects of direct intrastriatal administration of QUIN with the subsequent neurochemical and histological pathology reminiscent of Huntington's disease, many have proposed QUIN may indeed exert a causative role in this disorder via activation of NMDA receptors (Heng *et al.*, 2009), and has also been used as an animal model of Huntington's disease prior to the availability of genetic models. The disease is characterised by the progressive neurodegeneration of medium spiny neurons in the striatum, the primary region of disease pathology, while later progressing to other regions including: the basal ganglia, hippocampus, cortex and cerebellum, leading to CNS dysfunction and ultimately death (Roze *et al.*, 2008; Cowan and Raymond, 2006). Huntington's disease is a fatal disorder often emerging during midlife and is caused by a genetic mutation in the gene encoding the protein Huntington (Htt), where a codon bearing the amino acid sequence CAG repeats are elongated from the normal of 35 CAG codon repeats to over 100 (Rosas *et al.*, 2008). Furthermore, Huntington's disease pathology also displays an activated immune system thereby regulating kynurenine metabolites, accompanied by the subsequent loss of tryptophan (Leblhuber *et al.*, 1998). Chronic treatment with QUIN infused into the rat striatum precipitates deficits in spatial learning (Shear *et al.*, 1998), while an intrastriatal injection of QUIN in rodents induces lesions in the striatum reminiscent of Huntington's disease striatum (Beal *et al.*, 1990). Similarly, QUIN-induce lesions in the striatum of monkeys recapitulate the symptoms of dystonia and dyskinesia closely resembling those seen in Huntington's disease patients (Burns *et al.*, 1995). In addition to a range of behavioural deficits induced following even acute intrastriatal injections of QUIN (Bordelon and Chesselet, 1999; Schwarcz *et al.*, 2009), alterations in brain neurochemistry exist for substance P, GABA and glutamate (Nicholson *et al.*, 1995), while expression of the gene coding for Huntington's disease (Htt) is induced within 6 hours of QUIN-induced lesions (Carlock *et al.*, 1995). While these studies support the theory that elevation of QUIN could be related to deficits observed in Huntington's disease, intriguingly, early reports showed levels of QUIN remained unchanged in the CSF of patients suffering from Huntington's disease (Heyes *et al.*, 1992a, 1992b). While this implies QUIN

may not be involved in precipitating neuronal damage in Huntington's disease, it is likely that changes are occurring at highly localised and variable regions beyond the detection of gross level measurements. Interestingly, in a genetic mouse model of Huntington's disease (R6/2), levels of QUIN, 3-HK, IDO and KMO activity was increased in the cortex and striatum at 1-4 months of age (Guidetti *et al.*, 2006; Slow *et al.*, 2003; Sathyasaikumar *et al.*, 2010) and provided a good model of the earlier symptoms of Huntington's disease. This suggests QUIN may be important in the early prognosis of the disease, possibly accounting for the lack of QUIN detected in the CSF of patients at later stages. This is further supported by heightened levels of 3-HK and QUIN of up to 3- to 4-fold increases measured in the brains of patients with low grade Huntington's disease, while remaining unchanged in higher grade cases, suggesting KMO inhibition may indeed represent an attractive drug target (Giorgini *et al.*, 2005). Furthermore, while QUIN may not appear to be dysregulated in later stages of Huntington's disease, there is considerable evidence demonstrating its antagonist, KYNA is changed. Reduced levels are found in the caudate nucleus, while both KAT I and KAT II enzyme isoforms are reduced in the striatum of Huntington's disease patients (Jauch *et al.*, 1995). The significance here is that while QUIN remains unchanged, KYNA may have antagonised the effects of QUIN during physiological levels, thereby producing no net effect. However, since KYNA is substantially reduced in the striatum during later stages of Huntington's disease, this may result in the loss of KYNA-induced inhibition of QUIN, therefore physiological and non-toxic levels of QUIN may exert toxicity. Under basal resting levels this would prove difficult since physiological levels of QUIN remain non-toxic, however, since 3-hydroxykynurenine (3-HK), an intermediary metabolite of KYN metabolism before being metabolised further to QUIN, is increased in the cortex and striatum of Huntington's disease patients (Pearson and Reynolds, 1992; Guidetti *et al.*, 2006), this may produce a synergistic action with QUIN to induce toxicity. This is supported by *in vitro* studies demonstrating 3-HK to be neurotoxic and to potentiate the neurotoxic effects of QUIN even when non-toxic concentrations of QUIN are applied to cultures that failed to produce toxicity on its own, while another study demonstrated a similar effect in the rat striatum (Guidetti and Schwarcz, 1999). While QUIN mediates its neurotoxicity via activation of NMDA receptors and by generation of free radicals, 3-HK largely mediates its neurotoxicity through generation of reactive oxygen species (ROS) as opposed to activation of glutamate receptors (Okuda *et al.*, 1998).



### 1.7.1.3 QUIN-Induced Damage in Alzheimer's Disease

The contribution of QUIN has been assessed in Alzheimer's disease since this neurodegenerative disorder is also accompanied by a self-perpetuating inflammatory response where non-steroidal anti-inflammatory therapy is associated with improvements in symptomology. Recent studies have confirmed an increase in the activity of IDO with the subsequent increase in QUIN found in amyloid plaques, neurofibrillary tangles and dystrophic neurons (Guillemin *et al.*, 2005; Bonda *et al.*, 2010), while another study found QUIN to be an important regulator of astroglial dysfunction producing effects consistent with astrogliosis (Ting *et al.*, 2009), all pathological hallmarks of Alzheimer's disease. QUIN present in fatty acid deposits may contribute towards their propensity to cause toxicity in neurons via increasing oxidative stress which heavily surrounds amyloid plaques. Accumulation of QUIN within plaques is consistent with a recent report demonstrating QUIN induced phosphorylation of tau, a protein known to aggregate during plaque formation, in a dose-dependent manner via activation of NMDA receptors since this effect was abrogated by the pharmacological blockade of the receptor with memantine (Rahman *et al.*, 2009). Furthermore,  $\beta$ -amyloid proteins also appear to induce IDO activity (Walker *et al.*, 2006). These studies provide considerable evidence that Alzheimer's disease is associated with heightened levels of QUIN. Conversely, its antagonist KYNA is significantly reduced in the CSF and blood plasma (Vamous *et al.*, 2009; Hartai *et al.*, 2007) and therefore unable to antagonise the toxic effects of QUIN, leading to the proposal that reducing QUIN while simultaneously increasing KYNA concentration may provide an optimum mechanism for neuroprotection and be associated with improvements in neurological outcome (Gong *et al.*, 2011).

### 1.7.1.4 Cognitive Decline Associated with Degenerative Disorders

While many studies have linked heightened levels of QUIN with neurodegeneration in AIDS-dementia complex, Huntington's, Parkinson's and Alzheimer's disease, it is proposed this may directly contribute towards cognitive decline. This is supported by studies demonstrating the subchronic infusion of QUIN for 2 weeks into the cerebral ventricles precipitates deficits in memory and a significant loss of neurons in the basal forebrain (Miszta *et al.*, 1996). This is thought to resemble the underlying basis of cognitive decline associated with ageing, which is also a risk factor for neurodegeneration. Since CSF and brain levels of kynurenines (Wada *et al.*, 1994) including QUIN increase with age progression, it is possible the additional heightened levels of QUIN in neurodegenerative disorders, may serve to accelerate the process of age-related cognitive decline. This may potentially contribute to the early symptoms of cognitive decline appearing in patients suffering from these diseases.

#### 1.7.1.5 Mechanisms of Damage by Quinolinic Acid (QUIN)

Although QUIN-induced neurotoxicity via activation of NMDA receptors are likely to produce substantial damage, there exist other mechanisms by which QUIN induces neurotoxicity. Cytokines potentiate the effects of glutamate receptors, while the neurotoxicity exerted by activated macrophages are significantly attenuated by NMDA receptor antagonists (Ma *et al.*, 1997). This may likely involve the production of nitric oxide (NO) (Kim *et al.*, 1997) in mediating this potentiation, since the pro-inflammatory cytokine INF- $\gamma$  can induce the expression of inducible nitric oxide synthase (iNOS). Interestingly, QUIN has also been shown to induce iNOS expression thereby generating neurotoxic levels of nitric oxide. QUIN and INF- $\gamma$  may induce the expression of iNOS separately or INF- $\gamma$ -induced expression of iNOS may be secondary to the induction of IDO with the subsequent expression of QUIN. Another neurotoxic mechanism involved in many disorders, including Parkinson's disease, is the propensity of QUIN to induce the progressive dysfunction of mitochondria (Bordelon *et al.*, 1997), a process intimately linked with neurodegeneration (Lo Bianco *et al.*, 2004; Yasuda *et al.*, 2007; Rothfuss *et al.*, 2009). QUIN-induced lesions and neurotoxicity are attenuated significantly by reactive oxygen species (ROS) scavengers (Nakai *et al.*, 1999; Santamaría *et al.*, 2003), implying QUIN-induced toxicity is also mediated in part by oxidative damage produced by ROS in addition to its actions at the NMDA receptor.

### 1.8 Drug Development and the Kynurenine Pathway

#### 1.8.1 Kynurenic Acid (KYNA) Analogues

Since the kynurenine pathway is intimately involved in neurodegenerative disorders, often contributing towards disease progression, it has been the attention of intense interest to develop drugs capable of correcting kynurenine metabolites and to increase the levels of KYNA to mediate neuroprotection. For many neurodegenerative diseases, QUIN is believed to contribute towards neurodegeneration by inducing neuronal toxicity either directly via activation of NMDA receptors, synergistically with the actions of 3-HK or via the generation of ROS and induction of mitochondrial damage. Since KYNA can antagonise many of the neurotoxic effects of QUIN, initial studies led to the development of KYNA derivatives as a mechanism to elevate levels of KYNA. Indeed, KYNA and its derivatives protect against neuronal damage in primary neuronal cultures exposed to excitotoxins (Maroni *et al.*, 1992), while studies in stroke and ischemia show reductions in infarct volume following middle cerebral artery occlusion in rats (Chen *et al.*, 1993) and a decrease in cell death of hippocampal pyramidal neurons following transient carotid occlusion in gerbils (Pellegrini-Giampietro *et al.*, 1994). Due to relatively high doses being used in these studies, reflecting their poor

penetration of the BBB, several alterations and substitutions have been made to enhance their level of permeability with some success.

### 1.8.2 *Kynurenic Acid (KYNA) Prodrugs*

Later developments focused on an entirely different approach where compounds were generated acting as prodrugs to transport KYNA into the brain (Moore *et al.*, 1993). Such compounds including: L-4-chlorokynurenine and 4,6-dichlorokynurenine (Hokari *et al.*, 1996) show neuroprotective activity against QUIN-induced damage (Wu *et al.*, 2000). These developments have led to the patenting of a variety of agents used for the treatment of CNS disorders including: ischemic brain injury, stroke and epilepsy, thereby increasing central KYNA concentration to inhibit the excitotoxic activation of NMDA receptors. The major advantage of a therapy aimed at inhibiting the glycine binding site is they do not produce the serious neurotoxic and psychological side effects accompanied by the pharmacological blockade of NMDA receptors with MK-801 (dizocilpine), including disruptions in behaviour (Wood *et al.*, 1997), suggesting a kynurenine based therapy may likely be accompanied with safer symptomology profiles.

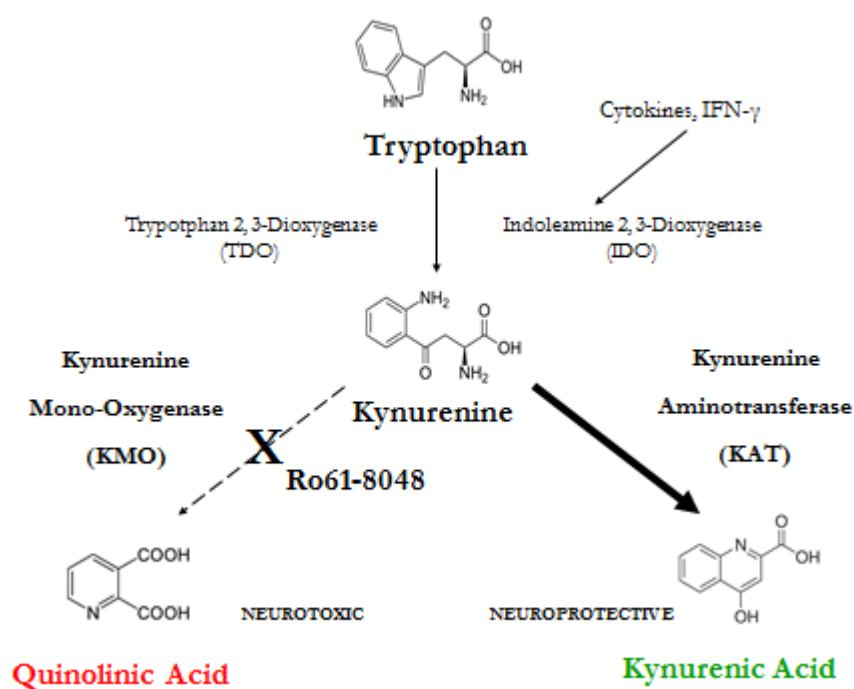
### 1.8.3 *Indoleamine 2,3-Dioxygenase (IDO) Inhibition*

Although previous therapies increase KYNA, they fail to produce alterations in neurotoxic levels of QUIN. To address the rise in QUIN following immune activation, IDO inhibition presents an obvious target since it is directly induced by pro-inflammatory cytokines and is responsible for the initial stage of tryptophan metabolism subsequently leading to the formation of QUIN. In addition to its role in neurodegeneration, IDO inhibition has been implicated as a novel therapeutic target for the treatment of cancer due to their tumor suppressive actions on T cells (Constantino, 2009; Muller and Scherle, 2006), leading to the subsequent patenting of many compounds inhibiting IDO (Andersen *et al.*, 2012).

### 1.8.4 *Kynurenine Monooxygenase (KMO) Inhibition*

Since many neurodegenerative disorders, including Alzheimer's disease, have increased QUIN while KYNA is reduced, alternative therapies aimed at reducing QUIN while simultaneously enhancing the production of KYNA were developed. Most attractive is the concept of KMO inhibition since neuroinflammation activates IDO stimulating tryptophan metabolism to form the precursor protein KYN. During neuroinflammation, KMO are the predominant enzymes responsible for the metabolism of KYN into QUIN, however, KMO inhibition permits the subsequent elevation of KYN allowing its transamidation to KYNA (figure 1-3). Within the context of neurodegeneration, such a mechanism deals with the

problem of enhanced QUIN, and addresses the need to stimulate KYNA synthesis to mediate neuroprotection by antagonising glutamate receptors (Maroni *et al.*, 1991). Further benefits from KMO inhibitors arise from the principal location of KMO in endothelial cells, infiltrating macrophages and microglia where it is localised to the outer membrane of mitochondria (Okamoto *et al.*, 1967) and responsible for the predominate QUIN production during infection. Therefore, KMO inhibitors would directly affect the ability of macrophage and microglial production of QUIN during neuroinflammatory diseases.



**Figure 1-3. The proposed effect of kynurenine monooxygenase inhibitors.**

While tryptophan is metabolised forming the precursor protein kynurenine (KYN), compounds capable of inhibiting its subsequent metabolism along the quinolinic acid arm of the pathway increases the concentration of KYN thereby subsequently allowing for the increased transamination of KYN into kynurenic acid (KYNA). A compound efficacious in inhibiting kynurenine monooxygenase (KMO) includes the experimental compound Ro61-8048, thereby elevating central concentrations of KYN and KYNA. Such an approach has been useful in treating hyperexcitability disorders like ischemia, stroke and epilepsy, while also proving useful in neurodegenerative disorders including Parkinson's and Huntington's disease.

#### 1.8.4.1 Kynurenine Monooxygenase (KMO) Inhibition: 'Proof of Concept'

The concept of blocking QUIN synthesis while diverting KYN metabolism to KYNA was first illustrated with the compound nicotinylalanine, a KMO inhibitor (Connick *et al.*, 1992; Russi *et al.*, 1992), co-administered with L-kynurenine and probenecid, to prevent elimination of KYNA from the brain via acidic transporters (Nozaki and Beal, 1992), elevated brain KYNA substantially by 5-fold with the subsequent prevention of seizures. Similarly, alanine derivatives including *meta*-nitrobenzoyalanine in mice sensitive to audiogenic seizures increased KYNA in the hippocampus correlating with seizure suppression (Chiarugi *et al.*, 1995), while another study showed protection against ischemic neuronal damage in gerbils (Cozzi *et al.*, 1999). Since *m*-nitrobenzoyalanine also offers protection against kainate-induced hippocampal damage, this further supports the theory that neuronal damage, caused by cerebral states of hyperexcitability in response to neuronal injury and ischemia, is mediated by QUIN produced from macrophages and microglial cells. Together, these studies confirm the hypothesis that KMO inhibitors are of therapeutic interest to reduce cerebral hyperexcitability and excitotoxic damage induced by excess QUIN. This concept has been extended to neurodegenerative models in subsequent studies where nicotinylalanine provided protection against NMDA or QUIN-induced damage in the rat striatum (Harris *et al.*, 1998), while co-administration of nicotinylalanine with L-kynurenine and probenecid protected nigrostriatal neurons against neurotoxicity induced by the local injection of QUIN or NMDA (Miranda *et al.*, 1999), confirming the potential use of KMO inhibitors in Huntington's and Parkinson's disease. The therapeutic utility of KMO inhibitors is further strengthened since *meta*-nitrobenzoyalanine not only increases KYNA and reduces QUIN, but also reduces other neurotoxic metabolites like 3-HK in the brain, which is known to produce synergistic neurotoxic actions with QUIN.

#### 1.8.4.2 Kynurenine Monooxygenase (KMO) Inhibitor Ro61-8048

Due to the relatively low potency of alanine derivatives, experimental compounds like Ro61-8048 were developed as a highly potent inhibitor of KMO, while remaining effective following oral administration in gerbils, raising KYNA concentrations in the extracellular fluid of the brain (Rover *et al.*, 1997). Since an induced transient ischemia in gerbils results in dramatic increases in QUIN, remaining elevated within ischemic regions for several days (Saito *et al.*, 1993), the therapeutic potential of Ro61-8048 was examined in organotypic hippocampal slice cultures of oxygen and glucose deprivation and in an *in vivo* stroke model in gerbils, to find Ro61-8048 increased central KYNA accompanied by attenuated levels of post-ischemic cell death (Carpenedo *et al.*, 2002; Rover *et al.*, 1997). Furthermore, neuroprotection provided was ineffective when 3-HK and QUIN were applied to hippocampal slice cultures

demonstrating Ro61-8048 exerts neuroprotection by reducing the synthesis of 3-HK and QUIN. These results have been extended in an *in vivo* model of cerebral ischemia where Ro61-8048 substantially reduced the degree of hippocampal cell death in rats accompanied by a 7.5-fold increase in KYNA (Cozzi *et al.*, 1999).

#### 1.8.4.3 KMO Inhibition by Ro61-8048 in Parkinson's Disease

Parkinson's disease is accompanied by an increase in QUIN and neuronal activity contributing towards the loss of dopamine function, however, a major problem facing dopaminomimetic therapy for Parkinson's disease, including patients suffering from focal dystonia, are presented by their adverse effects on inducing dyskinesia. Sufficient evidence indicates striatal alterations are involved in producing dystonic symptoms characterised by sustained muscle contractions leading to repetitive movements or abnormal postures (Zhuang *et al.*, 2004). Studies show drugs blocking NMDA receptors prevent levodopa (L-DOPA)-induced motor symptoms in animal models of PD (Blanchet *et al.*, 2004; Gregoire *et al.*, 2008), while another study demonstrated in both the 6-hydroxydopamine (6-OHDA) rodent model and 1-methyl 4-phenyl-2,3,6-tetrahydropyridine (MPTP) primate model of PD, that an inhibitor of glutamate release was efficacious in relieving or delaying L-DOPA-induced dyskinesias (Gilgum-Sherki *et al.*, 2003). While NMDA receptor antagonists can reduce the severity of parkinsonism in animal models and humans (Zipp and Fisher, 1993; Montastruc *et al.*, 1997), albeit with limited dosage due to their side effects, KYNA derivatives, acting at the glycine recognition site or KMO inhibitors, may be safer for use in clinical practice and could prove valuable adjuncts to the dopamine-based drugs currently available. Since NMDA receptor inhibitors are efficacious in alleviating dystonias and dyskinesias, and altered kynurenine metabolites are found in neurodegenerative basal ganglia disorders (Nemeth *et al.*, 2006), including patients with focal dystonia (Hartai *et al.*, 2007), Ro61-8048 was examined to correct changes in kynurenines while also inhibiting NMDA receptors to alleviate movement disorders. This may be useful since 3-HK is elevated in the putamen and substantia nigra of Parkinson's disease sufferers, accompanied with reduced KYN in the substantia nigra, putamen and frontal cortex, suggestive of increased KMO activity. A reduction in KYN also implies the reduced availability for KYNA synthesis, a combination of effects potentially contributing towards neuronal damage (Ogawa *et al.*, 1992). Consistently, Ro61-8048 reduces neuronal damage and dyskinesias in animal models of PD (Gregoire *et al.*, 2008) and in MPTP monkeys when co-administered with L-DOPA (Ouattara *et al.*, 2009). Systemic injection of Ro61-8048 increases extracellular concentrations of KYNA in different brain regions of mutant hamsters at anti-dystonic doses (Richter and Hamann, 2003), while intrastriatal injections significantly reduces the severity of dystonia in mutant hamsters (Hamann *et al.*,



2008). The therapeutic efficacy of Ro61-8048 in these models further strengthens the concept that KYNA plays an important role in basal ganglia function capable of reducing extracellular dopamine and glutamate in the striatum (Maroni *et al.*, 2005; Wu *et al.*, 2007). Consistently, systemic administration of Ro61-8048 in rats significantly increased KYNA in dialysates obtained from the parietal cortex, caudate nucleus and hippocampus while glutamate was reduced in dialysates collected from extracellular spaces of the caudate nucleus (Maroni *et al.*, 2005). Intrastriatal infusion of low concentration KYNA reduced extracellular levels of glutamate and dopamine in the rat striatum via inhibition of  $\alpha$ -7 nicotinic acetylcholine receptors located on glutamatergic afferents (Rassoulpour *et al.*, 2005; Wu *et al.*, 2007). This suggests another mechanism accounting for the anti-dystonic efficacy of Ro61-8048 and improvements to dyskinesias in parkinsonian monkeys following treatment with levodopa (Samadi *et al.*, 2005).

#### 1.8.4.4 Benefit of KMO Inhibition Over NMDA Receptor Blockers

A major benefit for modifying the kynurenine pathway as a drug target for CNS diseases involving neuroinflammation is since selective inhibition of kynurenine enzymes, including KMO, aimed at reducing QUIN while stimulating KYNA synthesis, would produce localised actions only affecting brain regions displaying neuroinflammation, while areas with normal glutamate signalling would remain unaffected, ultimately producing a safer side effects profile than NMDA channel blockers (Javitt & Zukin, 1991; Krystal *et al.*, 1994; Harris *et al.*, 2003; Kawabe *et al.*, 2007; Kawabe & Miyamoto, 2008). This is especially important considering every NMDA receptor channel blockers developed for treatment of stroke, ischemia, epilepsy or neurodegeneration have consistently failed in clinical trials due to their side effects greatly limiting their therapeutic potential. Indeed, this may be the principal reason supporting the continuous rise in interest of kynurenine pathway inhibitors, especially for KMO (Cesura and Rover, 1999), leading to the patenting of many compounds (Daily *et al.*, 2010; Zisapel *et al.*, 2012) for a wide range of CNS diseases including stroke and Huntington's disease (Muchowski and Giorgini, 2009; Muchowski *et al.*, 2011).

## 1.9 Purpose of Study

Many studies have demonstrated a range of behavioural and cognitive deficits in rodent offspring during adulthood when exposed to infectious or immune stimulating agents prenatally, however, the molecular changes underlying these behavioural and cognitive abnormalities remain poorly understood. Therefore, the first part of this project examined any changes in protein expression of a selected group of target proteins in embryo and neonatal brains that may be responsible for the emergence of schizophrenia-like symptoms during adulthood. Indeed, it remains within an appreciable understanding of the molecular alterations underlying the heightened risk for schizophrenia that would permit suitable therapies to follow.

Studies of maternal immune activation have assessed damage attributed to pro-inflammatory cytokines, while specific neuronal and behavioural deficits have been linked with particular members of the cytokine family. However, since maternal immune activation is accompanied with the subsequent activation of the kynurenine pathway, it is likely that kynurenine metabolites, including QUIN and 3-HK among others, are involved in mediating considerable amounts of this damage. Since KMO inhibitors provide neuroprotection against neuroinflammatory conditions where neurotoxicity is mediated through actions of QUIN and 3-HK, it was initially hypothesised that deficits in brain and behaviour, following maternal immune activation, may be reduced or prevented by selectively modulating the kynurenine pathway. However, it is appreciated that very little is known with regards to the physiological activity of the kynurenine pathway during normal foetal development, or its potential consequences of altering kynurenine metabolites on the offspring later in life, all factors which should clearly need to be addressed. For this reason, the project sought to examine the effects of disrupting the normal physiology of the kynurenine pathway *in utero* and its effects on brain development. Such findings would permit informed decisions for the potential utility of such a therapy in treating the effects of maternal immune activation.

On the other hand, many CNS disorders during adulthood involve changes in kynurenine metabolites contributing towards disease progression, either induced by infection, stress or other factors. This raises the possibility whether changes in kynurenines induced by disruptions in the pathway early in life, or even prenatally by conditions affecting the pregnant mother, may affect cognitive and behavioural functions in the offspring later in life (Stone and Darlington, 2013). Since environmental factors predispose individuals to heightened risks of developing CNS disorders, when exposed to subsequent life events, involves the activation of tryptophan metabolism along the kynurenine pathway, either in response to stress or



infection, this may be the common factor in mediating changes in glutamate receptor function. This suggests alterations in kynurenine metabolites, by modifying the activity of the kynurenine pathway, during development may also lead to subsequent cognitive and behavioural deficits in the offspring. Therefore, this project largely assessed the role of modifying the activity of the kynurenine pathway prenatally, using the KMO inhibitor Ro61-8048, on brain development in the offspring to identify whether prenatal disruption of kynurenines could predispose the offspring to subsequent risks in psychopathology during adulthood.

## Aims of the Project

### Poly(I:C) Model:

1. To assess the acute effects of maternal exposure to poly(I:C) on the protein expression of selected markers in embryo brains
2. To assess the effects of maternal immune activation during development and its impact on neonatal brains by examining alterations in protein expression of adolescent rats

### Ro61-8048 Model:

1. To assess the acute effects of prenatal exposure to Ro61-8048 on the protein expression of selected markers in embryo brains
2. To assess the impact of prenatal exposure to Ro61-8048 during development and its effects on neonates by examining alterations in protein expression in the brains of adolescent rats
3. To assess the impact of prenatal exposure to Ro61-8048 during development and its effects on young adult rats by examining alterations in protein expression in the hippocampus, cortex, midbrain and cerebellum
4. To assess changes in excitatory (VGLUT-1/2) and inhibitory (VGAT) transporters in the stratum pyramidale layer of the CA1 hippocampus of young adult rats exposed to Ro61-8048 prenatally
5. To assess changes in co-localisation between VAMP-1 and VGLUT-1/2 or VGAT in the stratum pyramidale layer of the CA1 hippocampus of young adult rats exposed to Ro61-8048 prenatally
6. To assess morphological changes of dendritic spine densities in apical and basal pyramidal cell dendrites in the CA1 hippocampus of young adult rats exposed to Ro61-8048 prenatally

## 2.0 Animals

All animal studies were carried out with ethical approval in accordance to the Animals (Scientific Procedures) Act 1986 of the UK, which was regulated and monitored by the Home Office, and all experimental procedures were approved by the University of Glasgow Research Ethics Committee. Male and female Wistar rats (Harlan Olac, UK) were housed together for mating in our in-house Central Research Facility (CRF), and monitored daily for the vaginal plug. Once successfully mated, pregnant females were housed alone from that point with free access to food and water.

## 2.1 Poly(I:C) Injection Schedules

### 2.1.1 Selected Dosage

Timing schedules and treatment dosages were carefully selected after a thorough review of the literature and a large number of test experiments performed by Dr. Caroline Forrest. Since adversely sick animals display overt signs of stress and abnormal behaviour towards neonatal pups after birth, factors that could confound the interpretation of the results, 10 mg/kg was selected as a suitable dose that could be administered to pregnant dams on three separate occasions during late gestation without evoking any signs of sickness (hyperpyrexia, lethargy, hyper-sensitivity, anorexia) or abnormal behavioural changes in the mother. This provided confidence that changes observed in neonatal pups during adolescence or early adulthood were not caused by changes in the mothers behaviour towards her litter. Furthermore, to minimise variations in responses, all animal injections were administered between 9 am and 11 am. The individual contributions made by others during the course of the work can be seen in the table 2-1.

**Table 2-1. Table of contributions**

<b>Action Done</b>	<b>Performed by</b>
Animal Injections	Dr. Forrest, CRF staff
Mating of Animals	CRF staff
Killing of Animals	Dr. Forrest, CRF staff
Drug Preparations	Dr. Forrest, Khalil, Mazura
Paraformaldehyde Preparation	Khalil, Mazura, Robert, Christine
Animal Perfusions	Dr. Hughes, Dennis

### *2.1.2 Embryo Experiments: 5 h Post-Injection*

For poly(I:C)-treated animals, two series of experiments were performed. Firstly, an intraperitoneal injection of 10 mg/kg poly(I:C) was administered to pregnant dams on gestation day E18 and euthanized 5 h later with CO<sub>2</sub>. Embryos were removed, and whole brains were extracted for western blotting. Blood from the mother was also removed by cardiac puncture for ELISA experiments by Dr. Caroline Forrest. A primary reason for this was to validate our model of maternal immune activation following poly(I:C) treatment, showing the elected dose of 10 mg/kg was sufficient to induce activation of pro-inflammatory cytokines, a response typical of immune activation. Additionally, we sought to examine any immediate changes of protein expression in the embryo brains as a direct consequence to an immunological challenge.

### *2.1.3 Neonatal Experiments: Postnatal Day 21 Animals*

Secondly, in order to assess the effects on maternal infection during development and its impact on neonatal pups later in life, pregnant rats were injected with 10 mg/kg poly(I:C) at gestation days E14, E16 and E18. This triple injection schedule was selected to extend the temporal impact of maternal immune activation on the embryos. Following the injections, gestation was allowed to proceed normally with a full-term delivery. Neonates were allowed to survive until postnatal day 21 (P21), the day of weaning, then taken from the home cage and euthanized with an intraperitoneal (i.p) injection of sodium pentobarbitone. Brains were rapidly removed and separated into two cerebral hemispheres, placed on dry ice and stored at -80°C to be processed for immunoblotting.

## 2.2 Ro61-8048 Injection Schedule

### 2.2.1 *Selected Dosage*

A dosage of 100 mg/kg was used in experiments where Ro61-8048 was administered via an intraperitoneal injection to pregnant rats. The dose was chosen since previous studies identified it to be a suitable dose that can be administered repeatedly to the same animal with minimal ill effects in adult rats (Clark *et al.*, 2005; Rodgers *et al.*, 2009). This dose was tolerated well by our animals and displayed no sickness behaviour. In order to study the normal physiological role of the kynurenine pathway during development, and its impact on brain function following maternal disruption of the kynurenines, a series of experiments were performed across four time points with either a single or triple injection schedule.

### 2.2.2 *Embryo Experiment: 5 h Post-Injection*

Firstly, 100 mg/kg Ro61-8048 was injected via an intraperitoneal injection to pregnant dams at gestation day E18 and euthanized 5 h later with CO<sub>2</sub>. Embryos were removed, and whole brains dissected for western blotting. Blood from the mother was removed by cardiac puncture for HLPC measurements by Dr. Caroline Forrest, primarily to validate our model that the selected dose was sufficient to induce profound changes in the levels of kynurenic acid as expected. Additionally, we sought to examine any immediate effects of Ro61-8084 on protein expression in the embryo brains as a consequence to changes in the levels of kynurenine metabolites.

### 2.2.3 *Embryo Experiments: 24 h Post-Injection*

The second series of experiments maintained the same protocol where pregnant dams were injected with 100 mg/kg Ro61-8048 at gestation day E18. However, pregnant rats were euthanized 24 h later on gestation day E19 with CO<sub>2</sub>. Embryos were removed and whole brains extracted for immunoblotting.

### 2.2.4 *Neonatal Experiments: Postnatal Day 21 Animals*

For the third series of experiments, in order to examine the effects of the kynurenine pathway of tryptophan oxidative metabolism during development and its impact on neonates later in life, pregnant rats were injected with 100 mg/kg Ro61-8048 at gestation days E14, E16 and E18. This triple injection schedule across late gestation was selected to maximise and extend the period in which the activity of the kynurenine pathway was disrupted. Following treatment schedules, gestation was allowed to proceed normally leading to a full-term delivery. Neonates were allowed to survive until postnatal day 21 (P21), the day of weaning, then removed from the home cage and euthanized with an intraperitoneal injection of sodium

pentobarbitone. Brains were rapidly removed and separated into two cerebral hemispheres, placed on dry ice and stored at  $-80^{\circ}\text{C}$  until further processing by immunoblotting.

#### *2.2.5 Postnatal Experiments: Postnatal Day 60 Animals*

For some disorders like schizophrenia, the full spectrum of behavioural symptoms only become apparent later in life during adulthood. For this reason, many studies have focussed on behavioural analysis on adult rats. Therefore, the fourth series of experiments sought to examine the effects of Ro61-8084 on young adult rats. Timed pregnant rats were administered an intraperitoneal injection of 100 mg/kg Ro61-8048 on days E14, E16 and E18 of gestation, and then proceeded normally leading to a full-term delivery. Pups were allowed to survive until postnatal day 60 (P60), then removed from the home cage and used in a series of three different experimental protocols.

- Firstly, P60 animals were euthanized with sodium pentobarbitone, and brains were removed and separated revealing two cerebral hemispheres. While subtle changes in localised brain regions of some proteins of interest may not have been detected by overall gross examination of entire halves of the cerebral hemisphere, as examined in P21 animals, at P60 both hemispheres were regionally dissected into: cortex, hippocampus, midbrain and cerebellum in artificial cerebrospinal fluid (ACSF; 2.2 mM  $\text{KH}_2\text{PO}_4$ , 2.0 mM KCl, 25mM  $\text{NaHCO}_3$ , 115 mM NaCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , glucose 10 (Sigma, UK), distilled water ( $\text{dH}_2\text{O}$ )), placed on dry ice and stored at  $-80^{\circ}\text{C}$ . The principal reason for this was to further localise changes in protein expression by immunoblotting.
- Secondly, P60 animals were deeply anaesthetised using sodium pentobarbitone and perfused intracardially via the left ventricle with mammalian ringer solution to flush out blood for 1 min followed by 4 % paraformaldehyde (PFA) containing 0.2 M phosphate buffer (PB) at pH 7.2 for 20 min at a comparable perfusion pressure to match the systemic circulation. Perfusion efficiency and tissue fixation was monitored by the hardening of internal organs, the disappearance of the dark red colour of the liver to a light pink colour implying the removal of blood, and the rigidity of limbs and tail. Brains were removed following perfusion and post-fixed for 4 h at  $4^{\circ}\text{C}$ , then rinsed in 0.3 M phosphate buffered saline (PBS) and stored in 30 % sucrose solution at  $4^{\circ}\text{C}$  until cut for processing by immunocytochemistry.

- Thirdly, P60 animals were euthanized with sodium pentobarbitone, and brains were rapidly removed, briefly rinsed in dH<sub>2</sub>O and immersed in the impregnation solution for golgi experiments.

### 2.3 Control Animals

Since handling animals during injection schedules and the injections themselves are sources of stress and may produce slight physiological alterations, to remove any bias or confounding effect on the results, naïve animals were not used as a control. Instead, control animals were subjected to the same timed-injection protocol where 0.9 % sterile saline was administered into pregnant dams during gestation days E18 for embryo experiments and E14, E16 and E18 for P21 and P60 experiments.

### 2.4 Preparation of Drugs for Animal Injections

#### 2.4.1 0.9 % Saline

0.9 g NaCl (Sigma, UK) was dissolved in 100 ml dH<sub>2</sub>O. 0.9 % saline was then sterilised in the flow hood by passing the solution through a sterile syringe. Aliquots of 0.9 % sterile saline were stored at -20°C ready for animal injections. To ensure sterile conditions at all times, the flow hood was sterilised with ethanol prior to use, all laboratory equipment including pipettes, pipette tips, ependorph tubes and laboratory gloves were sterilised with ethanol first then introduced into the flow hood, and sterile techniques were used throughout to ensure no pipette tip made contact with the surface of the flow hood.

#### 2.4.2 10 mg/kg poly(I:C)

Poly(I:C ; P0913, Sigma, Poole, UK) was removed from the freezer 30 min prior to use and allowed to thaw at room temperature. 10 mg was dissolved in sterile saline (0.9 % NaCl) and heated to 50°C to ensure complete solubility. Since double stranded RNA dissociates at high temperatures, the drug was allowed to cool at room temperature allowing doublestranded RNA to re-anneal. Poly(I:C) was sterilised in the flow hood by passing the solution through a sterile syringe. Aliquots of sterile poly(I:C) were stored at -20°C ready for animal injections.

#### 2.4.3 100 mg/kg Ro61-8048

100 mg Ro61-8048 (purchased from Rover) was dissolved in 100 ml of 60 mM NaOH (240 mg in 100 ml 0.9 % saline) and vortexed to aid dissolving of the drug. The pH of Ro61-

8048 was adjusted to pH 7.6 using 1 M HCL, then sterilised in the flow hood by passing the solution through a sterile syringe. Aliquots of sterile Ro61-8048 were stored at -20°C ready for animal injections.

## 2.5 Experimental Protocol

### 2.5.1 *Western Blots*

#### 2.5.1.1 *Injection Schedule*

Timed pregnant rats were injected with saline, poly(I:C) or 100 mg/kg Ro61-8048 at E18 for embryo experiments and euthanized 5 and 24 h later with CO<sub>2</sub>, while for postnatal experiments pregnant rats were injected at E14, E16 and E18. Pups were allowed to survive until P21 or P60, and then euthanized with sodium pentobarbitone. A total of 5-6 animals (both male and female) were randomly selected across 3 litters per treatment group.

### 2.5.2 *Methods*

#### 2.5.2.1 *Sample Preparation*

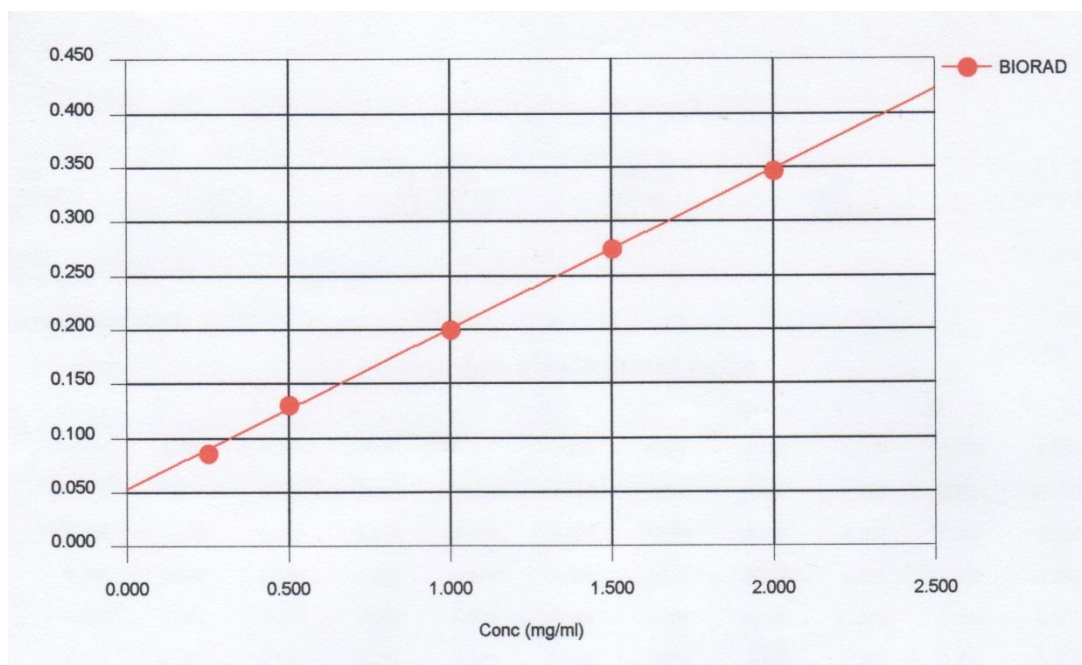
Frozen brain samples were weighed and multiplied by a factor of 5 to provide the total volume of RIPA buffer used to homogenise brain tissue. Protein was extracted from brain tissue by homogenising brain samples mechanically in glass test tubes containing RIPA buffer (60 mg TRIS, 87.6 mg NaCl, 10 mg SDS, 50 µl Triton X-100, 100 µl IGEPAL, 9850 µl sterile dH<sub>2</sub>O and a Roche complete protease inhibitor tablet ([Invitrogen](#))), while homogenates were placed in 1 ml ependorph tubes and centrifuged at 18,000 g for 5 min at 4°C, with the supernatants removed and aliquoted in 1 ml ependorph tubes and stored at -80°C for processing.

#### 2.5.2.2 *Bradford Protein Assay*

Total protein concentration in brain lysates were determined using the Bio-Rad Coomassie Blue protein assay ([Bio-Rad, Hamel Hampsted, UK](#)), based on the method of Bradford, where protein in brain lysates were compared against a standard curve of known protein content. Bovine Serum Albumin (BSA) standard (20 mg BSA and 100 ml dH<sub>2</sub>O) was diluted 1/10 with dH<sub>2</sub>O, and Biorad reagent was diluted with dH<sub>2</sub>O by ½. BSA standards were prepared in duplicates to produce the protein concentration curve using concentrations: 0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml and 2.0 mg/ml in universal tubes. BSA standards were diluted in dH<sub>2</sub>O to achieve these concentrations. Sample dilutions were made by a 1/10 dilution with dH<sub>2</sub>O followed by a 1/100 dilution with dH<sub>2</sub>O. 200 µl of diluted Biorad reagent was added to each standard and sample then vortexed. Finally, 200 µl of each



standard and sample was loaded onto a 96-well plate in duplicate, and absorbance was measured using an Opsys MR Plate Reader (Dyner Technologies, UK) at a wavelength of 595 nm using Revelation Quicklink Software (Dyner Technologies, version 4.25) and the BIORAD.asy program. A representative curve fitting from the Bradford protein assay is seen in figure 2-1. All measurements obtained from this assay were multiplied by a factor of 100 to compensate for the dilution factor applied earlier.



**Figure 2-1. BSA curve fitting from the Bradford protein assay.**

A representative example of a BSA standard curve obtained from the Bradford protein assay. The standard curve was used as a method of quantifying the amount of protein content in brain lysates, while a linear regression analysis was used to determine the line of best fit and to calculate the absorbance of brain lysates.

### 2.5.2.3 Gel Electrophoresis

Protein concentrations obtained from the Bradford assay were normalised and made up to equal working concentrations of known protein content ranging from 10-15 µg of protein and loaded onto gels. Protein samples (prepared as: 65 % protein sample, 25 % sample buffer and 10 % reducing agent (Life Technologies, Paisley, UK)) were heated in a water bath for 10 min at 70°C to denature the proteins. 10 µl SeeBlue pre-stained standard (Life Technologies, Paisley, UK) was loaded onto each gel, serving as a molecular weight marker, while 15 µl of the protein samples were loaded onto NuPAGE 4–12 % Bis-Tris (1.0 mm) 15-lane polyacrylamide gels (Life Technologies, Paisley, UK) to separate proteins

according to their molecular weight. 4–12 % gels were selected since many of our proteins of interest separate at a molecular weight range of 28–60 kDA, while 4–12 % polyacrylamide gels provide the greatest separation in this range. Gels were run in running buffer (50 ml NuPage MOPS/MES running buffer (Invitrogen), 950 ml dH<sub>2</sub>O) for the outer compartment and running buffer with 0.5 ml antioxidant buffer (Invitrogen) for the central compartment, and run at 150 V for 80 min at 240 mA for two gels in the unit.

#### 2.5.2.4 Gel Transfer

Separated proteins were transferred onto Invitrolon poly(vinylidene difluoride ; PVDF) membranes (Life Technologies, Paisley, UK). PVDF membranes were soaked in methanol for 1 min, then immersed and soaked alongside six sponges and filter paper in transfer buffer (50 ml NuPage MOPS/MES transfer buffer (Invitrogen), 1 ml sample antioxidant, 200 ml methanol for transferring two gels and 750 ml dH<sub>2</sub>O). Gel cassettes were cracked open exposing the inner polyacrylamide gel where a filter paper was placed over, with a PVDF membrane placed over the other side of the gel, and another filter paper placed over the PVDF membrane. Air bubbles were removed and each ‘membrane sandwich’ was placed in between two sponges at either side. Following this arrangement, each gel tank contained two PVDF membranes to transfer in between a total of 6 sponges that were held in place by the inner transfer chamber. This inner chamber was filled with transfer buffer (50 ml NuPage transfer buffer (Invitrogen), 1 ml sample antioxidant, 200 ml methanol for transferring two gels and 750 ml dH<sub>2</sub>O), while the outer compartment of the gel tank was filled with dH<sub>2</sub>O only. Gel transfer was set at 30 V for 70 min at 450 mA.

#### 2.5.2.5 Ponceu Staining

Uniformity of protein loading and the quality of gel transfer was ascertained by staining membranes with ponceu solution for 5 min on a shaker with slight agitation. The solution was then rinsed with dH<sub>2</sub>O to view the quality of protein bands and transfer efficiency. Membranes were then rinsed in 0.1 M sodium hydroxide (NaOH) to remove the staining effect of the solution, rinsed in dH<sub>2</sub>O, then washed in Tris-buffered saline containing 0.05 % Tween (TBST ; 100 ml 10 x TBS stock, 900 ml dH<sub>2</sub>O, 0.5 ml Tween-20, pH adjusted to 7.6 by adding drops of 18 M HCl).

### 2.5.2.6 Antibody Incubation

Membranes were blocked for 1 h at room temperature in 5 % milk solution (5 g non-fat dried milk (Invitrogen) dissolved in TBST), prior to overnight incubation at 4°C with the appropriate primary antibody (see table 2-2) prepared in 5 % milk-TBST solution incubated on a shaker with slight agitation. Membranes were then washed 3 times for 15 min with TBST, then incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (see table 2-3), prepared in 5 % milk-TBST solution, for 1 h at room temperature on a shaker with slight agitation. Following incubation with the secondary antibody, membranes were washed 3 times for 15 min each to remove excess unbound antibody.

**Table 2-2. Table of primary antibodies used in western blotting.**

Primary Antibody	Dilution		Source
	<i>Embryo Tissue</i>	<i>Postnatal Tissue</i>	
GluN1	1 : 500	1 : 1000	Millipore, UK
DISC-1	1 : 500	1 : 1000	Santa Cruz, UK
PSD-95	1 : 10,000	1 : 10,000	Cell Signalling, UK
TH	1 : 10,000	1 : 1000	Santa Cruz, UK
5HT-2 <sub>c</sub>	1 : 1000	1 : 1000	Santa Cruz, UK
A-Syn	1 : 1000	1 : 1000	Santa Cruz, UK
DCC	1 : 5000	1 : 5000	BD Biosciences, UK
Unc5-H1	1 : 1000	1 : 1000	Santa Cruz, UK
Unc5-H3	1 : 1000	1 : 1000	Santa Cruz, UK
SynT	-	1 : 5000	R & D Systems
SynPhy	-	1 : 40,000	Millipore, UK
VAMP-1	1 : 10,000	1 : 10,000	R & D Systems
RhoA	1 : 5000	1 : 1000	Santa Cruz, UK
RhoB	1 : 5000	1 : 1000	Santa Cruz, UK
DCX	1 : 20,000	1 : 1000	Santa Cruz, UK
Actin	1 : 20,000	1 : 1000	Santa Cruz, UK

**Table 2-3. Table of secondary antibodies used in western blotting.**

<b>Secondary Antibody</b>	<b>Dilution</b>	<b>Source</b>
Goat anti-mouse HRP	1 : 5000	Santa Cruz, UK
Goat anti-rabbit HRP	1 : 5000	Millipore, UK
Donkey anti-goat HRP	1 : 5000	Santa Cruz, UK

#### 2.5.2.7 Antibody Optimisation

To optimise the performance of antibodies in western blotting, the antibodies purchased were carefully selected after a review of the literature, and antibodies having most success in western blots and rat brain tissue were selected. All primary antibody dilutions were determined individually for each antibody by testing a range of dilutions on our samples based upon the published dilutions from other laboratories. Antibody dilutions that produced optimal signal strength with minimal background noise were selected for use. Once antibody dilutions were established for a particular set of samples, further optimisation was required for use in different brain regions at P60. Since some developmentally regulated proteins are more prevalent during development and decline with age, like DCX, RhoA and RhoB, antibody dilutions were also adjusted for experiments in embryo tissue, since the signal strength produced by the standard dilutions were either too strong or too weak.

#### 2.5.2.8 Chemiluminescence

HRP-conjugated secondary antibodies were visualised using Enhanced Chemiluminescence Plus (ECL plus ; 6 ml solution A, 150 µl solution B ) detection kit (GE Healthcare, UK) or Imobilon Western Chemiluminescent HRP Substrate (using equal volumes of luminol and peroxide solution ; Millipore, UK) for 5 min on the shaker shielded from light with slight agitation. Both chemiluminescent products oxidise the HRP substrate tagged to the secondary antibody, thereby emitting a signal from the membranes. Membranes were placed inside a cassette and exposed to radiographic x-ray films under red light and developed using a Kodak X-OMAT 2000 processor. Exposure times varied depending on the antibody used, its working dilution and signal strength, while the principal aim remained to achieve an optimal balance between signal-background ratios.

### 2.5.2.9 Data Analysis and Statistics

Western blots were quantified using Image J software (<https://rsb.info.nih.gov/ij/>) where optical densities of individual bands were measured. In order to control for variations in the total amount of protein loaded onto gels, the actin housekeeping gene was processed in parallel and examined. All results are expressed as a ratio taken of the intensity of the target protein to the intensity of the actin housekeeping gene. Statistical comparisons were made using Graphpad InStat software between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with poly(I:C) or Ro61-8048. Since multiple protein markers were examined in the same samples obtained from the same animals, a one-way ANOVA with Bonferoni correction for multiple comparisons was performed. All graphical data was generated using Prism 5 software.

## *2.5.3 Immunocytochemistry*

### 2.5.3.1 Injection Schedule and Perfusions

Timed pregnant rats were injected with 100 mg/kg Ro61-8048 at gestation days E14, E16 and E18. Pups were allowed to survive until P60, and then perfused with 4 % paraformaldehyde (PFA). Brains were removed and post-fixed for 4 h, then stored in 30 % sucrose. A total of 8 animals (both males and female) were randomly selected across 4 litters per treatment group for saline and Ro61-8048

### 2.5.3.2 Methods

Brains were cut into 60 µm thick coronal sections using a Leica VT1200 series vibratome (Leica, UK), where the inner chamber was filled with 0.1 M phosphate buffer (PB), and sections were collected in tubs containing 0.1 M PB. Sections were immersed in 50 % ethanol solution for 30 min to enhance antibody penetration, then rinsed 3 times 10 min each in 0.3 M phosphate buffered saline (PBS). Free-floating sections were incubated in a cocktail of primary antibodies (see table 2-4) for 72 h at 4°C with slight agitation. Sections were rinsed 3 times for 10 min each in 0.3 M PBS to remove excess unbound primary antibody, before being incubated in a cocktail of secondary antibodies (see table 2-5) overnight at 4°C with slight agitation. The secondary antibodies consisted of anti-goat, anti-rabbit and anti-mouse IgGs conjugated to Rhodamine Red, Dylight 649, Alexa 488 and Alexa 647 fluorphores. All antibody solutions were made up in PBS containing 0.3% Triton X-100 (PBST; Sigma, UK). Following incubation with the secondary antibodies, sections were shielded from light, rinsed 3 times for 10 min each in 0.3 M PBS, to wash away excess unbound antibody, and mounted in anti-fade vectorshield H-1000 medium (Vector Laboratories Inc, Burlingame, CA, USA) on

glass slides and stored at -20°C. Sections were then ready to be visualised with a confocal microscope.

**Table 2-4. Table of primary antibodies used in immunocytochemistry.**

Primary Antibody	Dilution	Source
VAMP-1	1 : 250	R & D Systems, UK
VGLUT-1	1 : 500	Synaptic Systems, Germany
VGLUT-2	1 : 500	Synaptic Systems, Germany
VGAT	1 : 1000	Synaptic Systems, Germany

**Table 2-5. Table of secondary antibodies used in immunocytochemistry.**

Secondary Antibody	Dilution	Source
Alexa 488 (donkey anti-goat)	1 : 500	Molecular Probes, UK
Dylight 649 (donkey anti-rabbit)	1 : 500	Molecular Probes, UK
Alexa 647 (donkey anti-rabbit)	1 : 500	Molecular Probes, UK
Rhodamine Red (donkey anti-mouse)	1 : 500	Jackson Laboratories, UK

### 2.5.3.3 Preparation of Buffers

Buffers and solutions used for immunohistochemistry were prepared as follows:

- 0.2 M PB (1120 ml solution A (37.44 g  $\text{NaH}_2\text{PO}_4(2\text{H}_2\text{O})$  (Fisher Scientific) in 1200 ml  $\text{dH}_2\text{O}$ ), 2880 ml solution B (84.90 g  $\text{Na}_2\text{HPO}_4$  (Fisher Scientific) in 3000 ml  $\text{dH}_2\text{O}$ ), and adjusted to pH 7.4.
- 0.3 M PBS (50 ml 0.2 M PB, 18 g NaCl (Fisher Scientific), 950 ml  $\text{dH}_2\text{O}$ )
- 4 % paraformaldehyde (PFA ; 40 g PFA (Sigma, UK) in 400 ml  $\text{dH}_2\text{O}$  heated to 68°C, a few ml 1 M NaOH to aid dissolving of PFA, all of which was done in the fume

hood, 500 ml 0.2 M PB, 100 ml dH<sub>2</sub>O to make it up to 1 L, then filtered through a watmans filter paper.

- Mammalian Ringer Solution (45 g NaCL (Sigma, UK), 2 g KCL (Sigma, UK), 1.25 g CaCL (Sigma, UK), 0.025 g MgCL (Sigma, UK), 2.5 g NaHCO<sub>3</sub> (Sigma, UK), 0.25 g NaH<sub>2</sub>PO<sub>4</sub> (Sigma, UK), 5 g glucose (Sigma, UK), 5 L dH<sub>2</sub>O).

#### 2.5.3.4 Confocal Microscopy and Image Acquisition

For the VAMP-1, VGLUT-1/2 and VGAT series of experiments, three to four hippocampal sections per animal were examined using a BioRad Radiance 2100 confocal laser scanning system equipped with lasers: Argon (488 nm), Green Helium Neon (543 nm) and Red Diode (637 nm), in conjunction with Laserssharp 2000 software. Pyramidal cell bodies in the stratum pyramidale of the CA1 region of the hippocampus were scanned using a 60 x oil immersion objective lens (numerical aperture 1.4, and an image size of 1024 x 1024) with a zoom factor of 3 (yielding a pixel size of 0.07 µm). Each field consisted of 30 optical sections at an increment of 0.3 µm z-separation. In an attempt to count the individual punctate staining for analysis, the contrast of each colour channel was manually adjusted within the maximum range to minimise the fusion of puncta. Individual punctate staining was manually counted in every third subfield in an area measuring 50 µm x 50 µm from the 1024 x 1024 pixel images. Each channel for VAMP-1, VGLUT-1/2 and VGAT was analysed separately to identify and count manually immunopositive puncta. Co-localisation of VAMP-1 with VGLUT-1/2 was analysed by merging the two channels together and manually counting the number of co-localised puncta. Co-localisation of VAMP-1 with VGAT was also measured in the same way. The criterion by which punctate staining was considered to be co-localised was when the overlap was complete or occupied most of the puncta, while also exhibiting morphological similarity. The manual counting of punctate staining was performed using ImageJ software.

#### 2.5.3.5 Statistical Analysis

Statistical comparisons were made using Graphpad InStat software, between groups of animals born to mothers injected with saline and groups of animals born to mothers treated with Ro61-8048. This protocol allowed the use of an unpaired *t*-test to examine differences between the two for each target protein of interest. A probability value of 0.05 was adopted as the working criterion for significance. All graphical data was generated using Prism 5 software.



## 2.5.4 Golgi Staining

### 2.5.4.1 Injection Schedule

Timed pregnant rats were injected with 100 mg/kg Ro61-8048 at gestation days E14, E16 and E18. Pups were allowed to survive until P60, and then euthanized with sodium pentobarbitone. Brains were rapidly removed, briefly rinsed in dH<sub>2</sub>O, then immersed in the impregnation solution. A total of 9 animals (both male and female) were randomly selected across 3 litters per treatment group for saline and Ro61-8048.

### 2.5.4.2 Methods

Golgi staining was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, USA) and tissue was processed according to the manufacturer's instructions. Freshly dissected rat brains were rinsed briefly in dH<sub>2</sub>O and immersed in the impregnation solution (in equal volumes of solution A and B prepared 24 h in advance) at room temperature for 2 weeks, while shielded from light. The impregnation solution was replenished after the initial 24 h of immersion. Brains were then removed from the impregnation solution, transferred into solution C and stored at 4°C for at least 48 h, while shielded from light. Solution C was replenished after the initial 24 h of immersion. Brains embedded in 4 % agar (4 g agar (Sigma, UK), 100 ml dH<sub>2</sub>O) were cut into 200 µm thick coronal sections using a Leica VT1200 series vibratome (Leica, UK), where the inner chamber was filled with solution C, and mounted onto gelatin-coated slides. Sections were dried at room temperature, while being shielded from light, rinsed in dH<sub>2</sub>O twice for 2 min each while renewing the dH<sub>2</sub>O after every wash, then stained in an equal mixture of solution D and E with twice the volume of dH<sub>2</sub>O (ratio of 1:1:2) for 10 min, then rinsed in dH<sub>2</sub>O 2 times for 4 min each, continually renewing the dH<sub>2</sub>O after every wash. Sections were dehydrated serially in 50 % ethanol for 4 min, 75 % ethanol for 4 min, 95 % ethanol for 4 min, followed by absolute ethanol 4 times for 4 min each, before being cleared in histoclear solution (ThermoFisher Scientific) 3 times for 4 min each, and a coverslip mounted using histomount (ThermoFisher Scientific).

#### 2.5.4.2.1 Method of Coating Glass Slides with Gelatin

Normal glass slides were inserted in slide racks and immersed in liquid detergent and left for 24 h to clean the slides of any bacteria or contaminant. Slides were rinsed in water repeatedly to remove the detergent then rinsed in dH<sub>2</sub>O, before being immersed inside dH<sub>2</sub>O containing cold fish skin gelatin (Sigma, UK) and chrome allum (Sigma, UK), an antibacterial agent used to prevent bacterial growth, and placed inside a drying cabinet to dry. Once dried, about 1 h later, slides were dipped inside the gelatin solution again and left to dry. This



procedure of immersing the slides in gelatin solution and being left to dry was repeated 3-4 times. Upon the final wash, slides were left to dry in a drying cabinet for 72 h before use.

#### 2.5.4.3 Microscopy

Secondary oblique dendrites on pyramidal neurons from the CA1 region of the hippocampus were observed by an investigator blind to the treatment group under a Nikon Eclipse E400 microscope where dendritic spines were traced and counted on a 2-dimensional plane by using a 100 x objective lens with oil immersion. Only fully impregnated neurons and dendritic spines, as ascertained by microscopic observation, in the absence of obscured dendritic branches due to neighbouring cells, blood vessels or precipitate were selected. To ensure relatively complete cells were used, the chosen cells were located close to the centre of the section while superficial cells, with significant processes cut at the surface of the section, were excluded from analysis. The identification of thick and thin spines were based upon the criterion of Harris et al., 1992, where spines were judged to be mushroom if the diameter of the head was greater than the diameter of the neck, while spines were judged to be thin if the length was greater than the neck diameter and if the diameter of the head and neck were similar. Dendritic spines were assessed while remaining within the same plane of focus for the analysis. 3 dendritic segments per cell and a total of 4 cells per animal from 9 animals per treatment group contributed towards the analysis.

#### 2.5.4.4 Statistical Analysis

Morphological data was analysed using an unpaired *t-test* to compare differences in the overall density of mushroom spines and thin spines between control and Ro61-8048-treated groups of animals. A probability value of 0.05 was adopted as the working criterion for significance. All graphical data was generated using Prism 5 software.

## 2.6 Parallel Studies

Since this project constituted towards a larger study within the laboratory, it is often required to discuss the results presented in this thesis in conjunction with results obtained from others within the laboratory. Therefore, throughout this thesis, where the discussion has involved the results produced from experiments performed by other individuals, they have been collectively referred to as ‘in a parallel study’. The individual contributions can be seen in the table 2-6. Cytokine and HPLC analysis was performed by Dr. Forrest to validate the efficacy of the two drugs employed in this study. While ELISA assays were used to confirm the ability of poly(I:C) to activate the immune response, HPLC analysis

was used to confirm the efficacy of the KMO inhibitor, Ro61-8048, in elevating the levels of KYNA in the CNS. Electrophysiological experiments were performed by Prof. Stone to investigate changes in synaptic plasticity and transmission in animals born to mothers treated with Ro61-8048. Furthermore, Mrs. Pisar performed experiments using the same experimental protocols and techniques, as presented within this thesis, but assessed the protein expression of a different set of molecular targets.

**Table 2-6. The contributions of others referred to ‘in a parallel study’.**

<b>Experiment</b>	<b>Performed by</b>
Cytokine analysis (ELISA) studies	Dr. Forrest
HPLC analysis	Dr. Forrest
Electrophysiology	Prof. Stone
Western blotting	Mrs. Pisar
Immunocytochemistry	Mrs. Pisar

### 3.1 Prenatal exposure to poly(I:C) in a rat model of maternal infection induces significant alterations in neurodevelopmental proteins widely associated with the emergence of schizophrenia symptoms

Since the initial report by Mednick (Mednick *et al.*, 1988) proposing an elevated risk in development of psychopathology in humans during adulthood born to mothers exposed to the influenza virus, several studies have replicated this finding with a variety of infectious agents. Conclusive serological evidence confirms the hypothesis that maternal exposure to a bacterial or viral infection during pregnancy can increase the risk of schizophrenia developing in the offspring during adulthood (Brown *et al.*, 2009). Animal models have been developed to understand the molecular mechanisms and pathophysiological alterations taking place within embryo brains that may be responsible for its abnormal development and subsequent onset of schizophrenia-associated symptoms. Studies using poly(I:C) and LPS have demonstrated a range of abnormalities in mice and rats closely resembling the endophenotype of schizophrenia, such as impairments in prepulse and latent inhibition, enhanced sensitivity to dopamine-stimulating treatment with amphetamine or to NMDA receptor blockade by dizocilpine (MK-801), and cognitive deficits including impairments in working memory and significantly reduced intelligence rates (Brown *et al.*, 2004; Buka *et al.*, 2008; Brown *et al.*, 2009; Meyer *et al.*, 2007). While many laboratories have focused on identifying behavioural abnormalities following infection *in utero*, and examining the efficacy of current anti-psychotics in alleviating these symptoms, few laboratories have assessed the molecular changes in protein expression taking place that may account for such observations. Indeed, if novel therapies are to be developed to correct for the abnormalities induced by infection or to prevent neurodevelopmental disorders like schizophrenia, importance falls upon identifying molecular alterations induced by infectious agents in either the mother, foetus or neonate, and elucidating how these changes may influence subsequent brain development. Since schizophrenia is not characterised as a neurodegenerative disorder, with the progressive loss of neurons, but increasingly becoming viewed as a neurodevelopmental disorder, with problems in synaptic communication and an abnormal wiring of cortical neurocircuitry, proteins known to be involved in all aspects of these processes including neurogenesis, cell growth and migration, axonal guidance and synaptogenesis were carefully selected and examined. Microarrays show genes coding for many of these processes are involved in the pathophysiology of schizophrenia (reviewed in Feldon and Folsom, 2009), further confirming the relevance of our selection of proteins that span a wide spectrum based upon their

developmental function, while taking into consideration their trajectory timescale of appearance and the quality of antibodies commercially available. To this end, we selected the ubiquitously expressed core subunit of the NMDA receptor, GluN1, DISC-1 and the Rho GTPases: RhoA and RhoB for their involvement in mediating many actions downstream of the NMDAR, while DISC-1 was selected for its association with schizophrenia. The netrin family of proteins: Unc5H1, Unc5H3 and DCC were selected for their role in axonal guidance and close relationship with dopaminergic neurons.  $\alpha$ -synuclein ( $\alpha$ -Syn) was examined for its role in Parkinson's disease and its close association with dopaminergic neurons, while dopaminergic and serotonergic transmitter systems were examined using tyrosine hydroxylase (TH) and the serotonin 5HT-2<sub>c</sub> receptor (5HT-2c) as molecular targets. Doublecortin (DCX) was used as a molecular marker of neurogenesis while synaptic proteins including: synaptophysin (SynPhy), synaptotagmin (SynT) and vesicle associated membrane protein (VAMP)-1 (synaptobrevin) were monitored as an indication of synaptic function.

Since many laboratories have consistently replicated behavioural deficits reminiscent of schizophrenia in both adult mice and rats when exposed to the viral mimetic poly(I:C) prenatally, including at the dosage used in our experiments, the aims of these experiments sought to investigate the immediate acute effects of prenatal exposure of poly(I:C) on the relative or absolute levels of protein expression in the embryo brains as a direct consequence to an immunological challenge. Therefore, 10 mg/kg poly(I:C) was administered via an intraperitoneal injection to pregnant dams on gestation day E18 and euthanized 5 h later with CO<sub>2</sub>. Embryos were removed, whole brains were extracted and western blotting was performed on brain lysates.

*3.1.1 Prenatal exposure to poly(I:C) in a rat model of maternal infection does not alter protein expression of markers in embryos at 5 h*

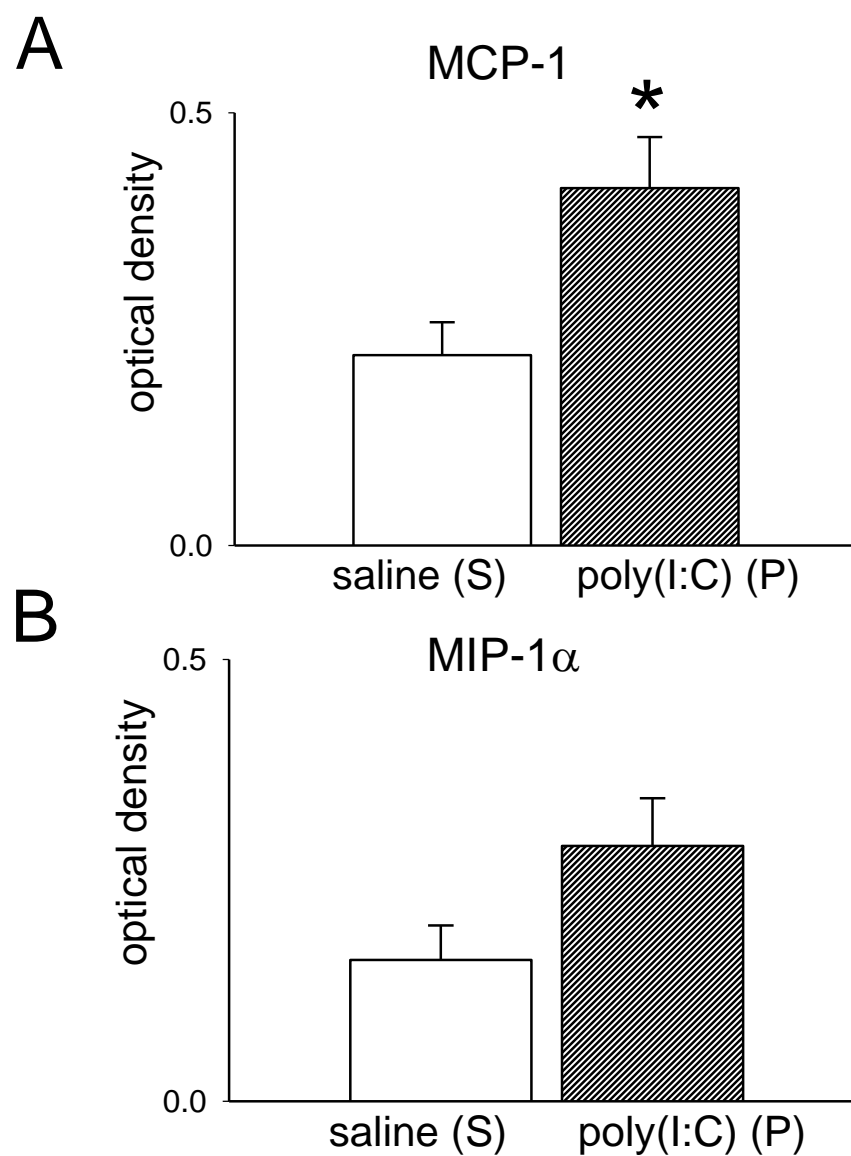
ELISA analysis in a parallel study showed the elected dose of 10 mg/kg poly(I:C) treatment induced activation of the maternal immune response since monocyte chemoattractant protein-1 (MCP-1) was significantly increased following treatment (figure 3-1)

The quantified protein expression from the western blots showed no change was observed in embryo brains 5 h post-treatment with poly(I:C) in the expression of GluN1, DISC-1, RhoA and RhoB ( $p > 0.05$ , figure 3-2).

When the netrin family of proteins were examined in the embryo brains, the quantified protein expression from the western blots showed no change was observed in Unc5H1, Unc5H3, and DCC ( $p > 0.05$ , figure 3-3).

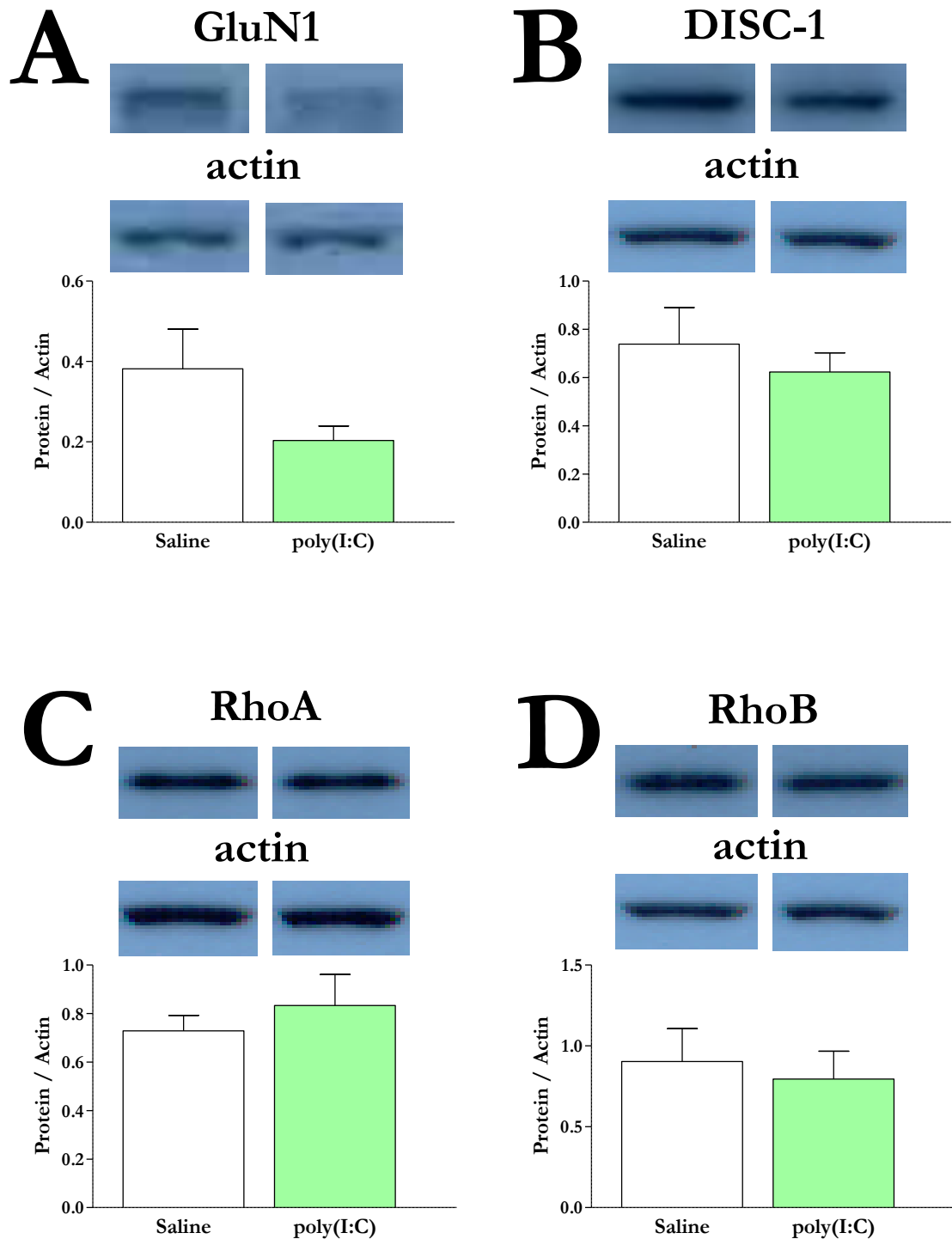
The quantified protein expression of tyrosine hydroxylase (TH), serotonin 5HT-2<sub>C</sub> receptor (5HT-2<sub>C</sub>) and  $\alpha$ -synuclein ( $\alpha$ -Syn) were unchanged ( $p > 0.05$ , figure 3-4).

Since synaptic markers are present in great abundance during development, in the acute experiments, only VAMP-1 (synaptobrevin) was examined to find no change in protein expression between treatment groups, while similarly no change was seen in the neurogenesis marker doublecortin (DCX ;  $p > 0.05$ , figure 3-5).

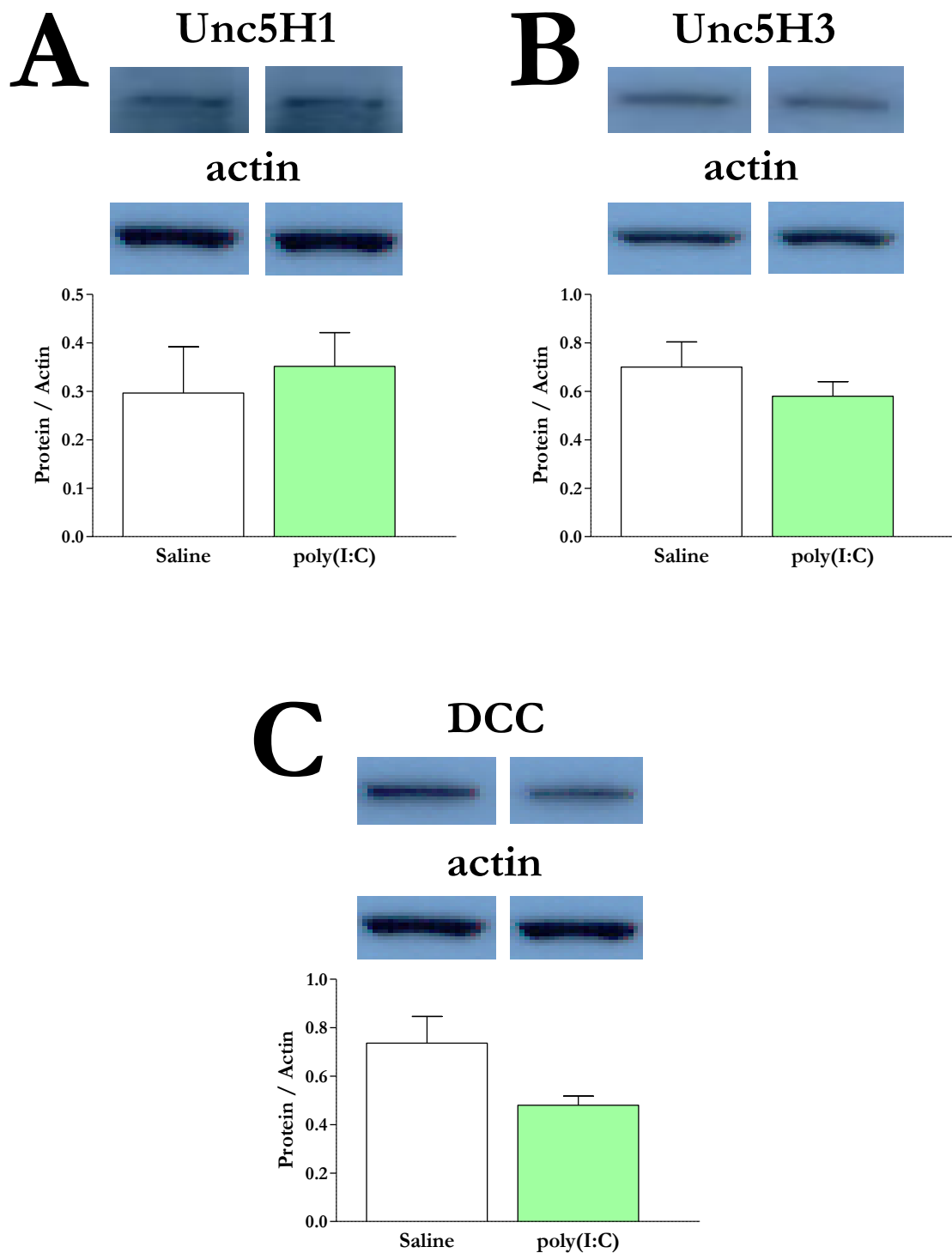


**Figure 3-1. Poy(I:C) increases the expression of MCP-1.**

Bar chart shows the maternal blood levels of (A) monocyte chemoattractant protein-1 (MCP-1) and (B) MIP-1 $\alpha$  measured at 5 h following poly(I:C) injection. Results are displayed as  $\pm$  s.e.mean ( $n = 3$ ) in arbitrary units of optical density. Statistical comparisons were made using an unpaired  $t$  test between groups of animals treated with saline and groups of animals treated with poly(I:C). A probability value of 0.05 was set as the criterion for significance.



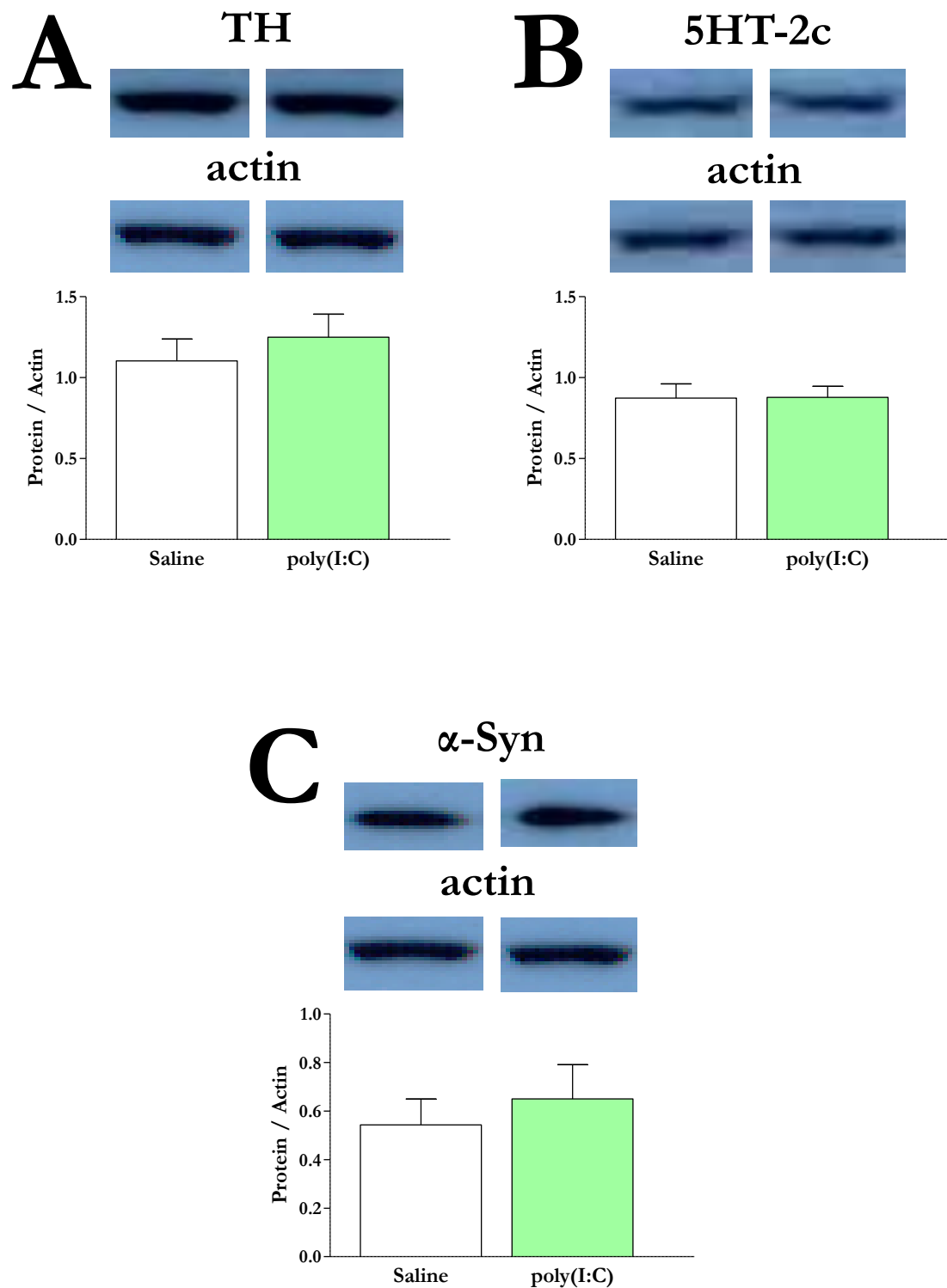
**Figure 3-2. GluN1, DISC-1, RhoA and RhoB expression in poly(I:C)-treated embryo brains.** Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1, (C) RhoA and (D) RhoB in the brains of the embryo following maternal exposure to 0.9 % saline or 10 mg/kg poly(I:C) on gestation day E18. Pregnant mothers were euthanized 5 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with poly(I:C). Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-3. Unc5H1, Unc5H3 and DCC expression in poly(I:C)-treated embryo brains.**

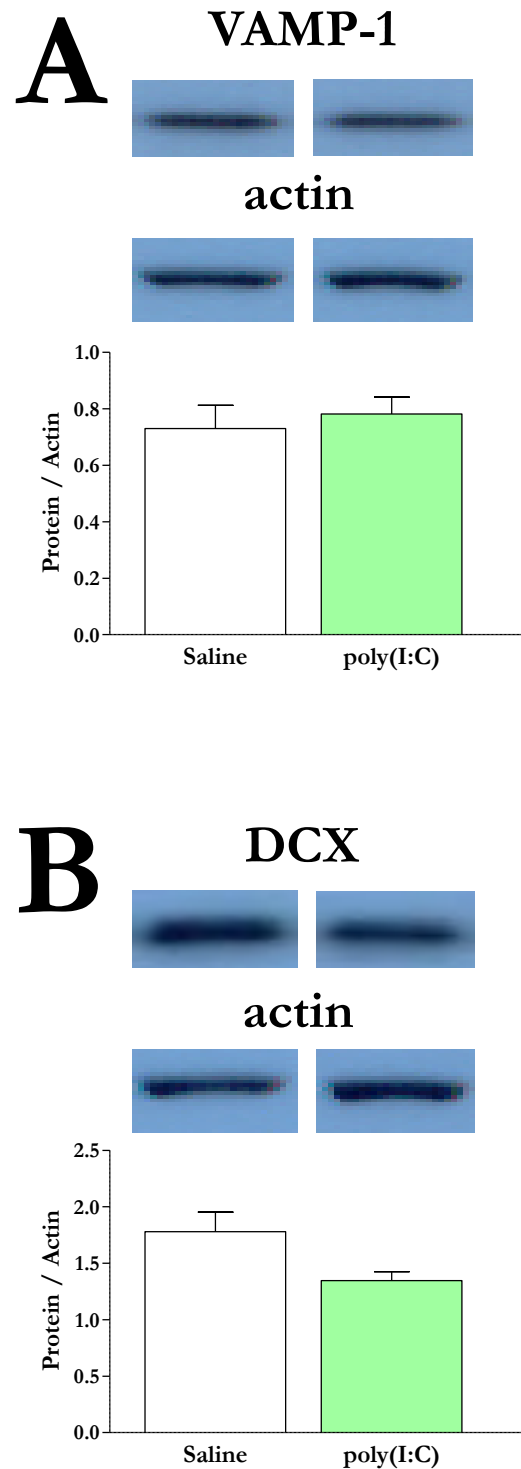
Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC, in the brains of the embryo following maternal exposure to 0.9 % saline or 10 mg/kg poly(I:C) on gestation day E18. Pregnant mothers were euthanized 5 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with poly(I:C). Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.





**Figure 3-4. TH, 5HT-2<sub>c</sub> and α-synuclein expression in poly(I:C)-treated embryo brains.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2<sub>c</sub> and (C) A-Syn in the brains of the embryo following maternal exposure to 0.9 % saline or 10 mg/kg poly(I:C) on gestation day E18. Pregnant mothers were euthanized 5 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with poly(I:C). Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-5. VAMP-1 and doublecortin expression in poly(I:C)-treated embryo brains.**

Bar charts illustrate the quantified protein expression of (A) VAMP-1 and (B) DCX in the brains of the embryo following maternal exposure to 0.9 % saline or 10 mg/kg poly(I:C) on gestation day E18. Pregnant mothers were euthanized 5 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with poly(I:C). Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.

### 3.2. Examination of Protein Markers in P21 Animals

Since poly(I:C) treatment in the pregnant mother induced no specific alterations in protein expression in the offspring at 5 h, this was compared with P21 animals. While the effects of cytokine activation during infection are relatively transient (Cunningham *et al.*, 2007), long-term consequences of an inflammatory challenge on protein expression were assessed in postnatal day 21 animals to potentially reflect the long-term deficits in behaviour following immune challenge. Furthermore, poly(I:C) is able to mimic the maturational delay in the onset of full blown schizophrenia symptoms, only emerging during early adolescence and progressively worsening until adulthood (Weinberger, 1987), suggesting poly(I:C) exposure *in utero* triggers a cascade of events persisting in postnatal animals. Therefore, it was hypothesised further deficits in neurodevelopmental proteins would be observed during later stages of development. To identify these changes, pregnant rats were injected with 10 mg/kg poly(I:C) at gestation days E14, E16 and E18 in order to extend the temporal impact of maternal immune activation on embryos. Gestation was then allowed to proceed normally with a full-term delivery, and animals were euthanized at P21 and brains were removed and separated into two cerebral hemispheres, then processed for western blotting.

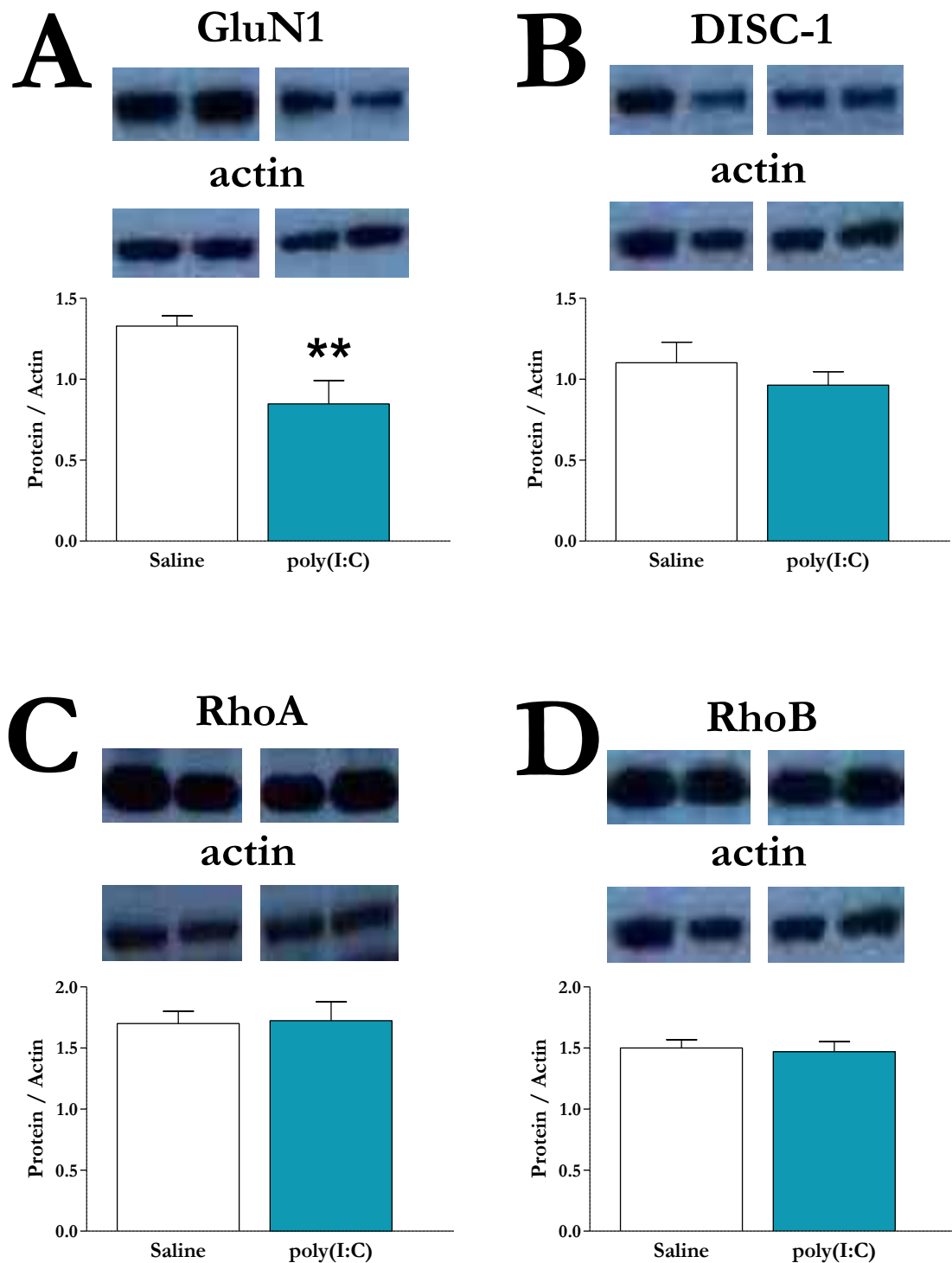
*3.2.1 Prenatal exposure to poly(I:C) in a rat model of maternal infection alters the expression of proteins relevant to schizophrenia in P21 neonatal brains*

When protein expression was compared in postnatal animals, DISC-1, RhoA and RhoB remained unchanged in the poly(I:C)-treated group, while there was a significant decrease in the protein expression of GluN1 ( $p < 0.01^{**}$ ) in groups of animals born to mothers treated with poly(I:C) prenatally (figure 3-6).

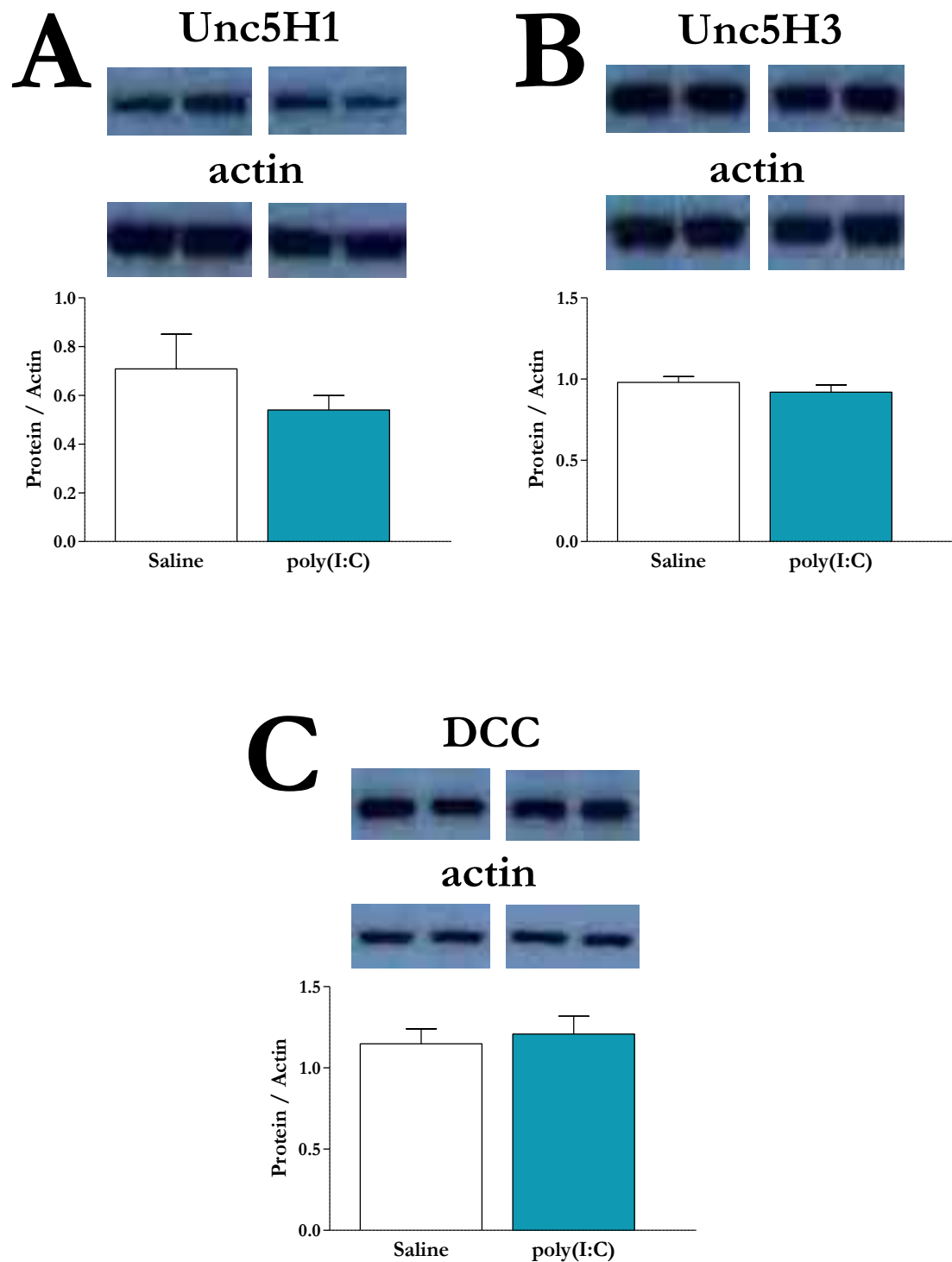
No significant changes were seen in the netrins including Unc5H1, Unc5H3 and DCC ( $p > 0.05$ , figure 3-7).

At P21, no changes were seen in DCX, TH, and 5HT-2<sub>c</sub>, while the protein expression of  $\alpha$ -synuclein ( $\alpha$ -Syn) was substantially reduced in groups of animals born to poly(I:C)-treated mothers ( $p < 0.01^{**}$ , figure 3-8).

When the protein expression of synaptic markers: VAMP-1 (synaptobrevin), synaptotagmin (SynT) and synaptophysin (SynPhy) were examined, no significant differences existed between treatment groups ( $p > 0.05$ ) as seen in figure 3-9.

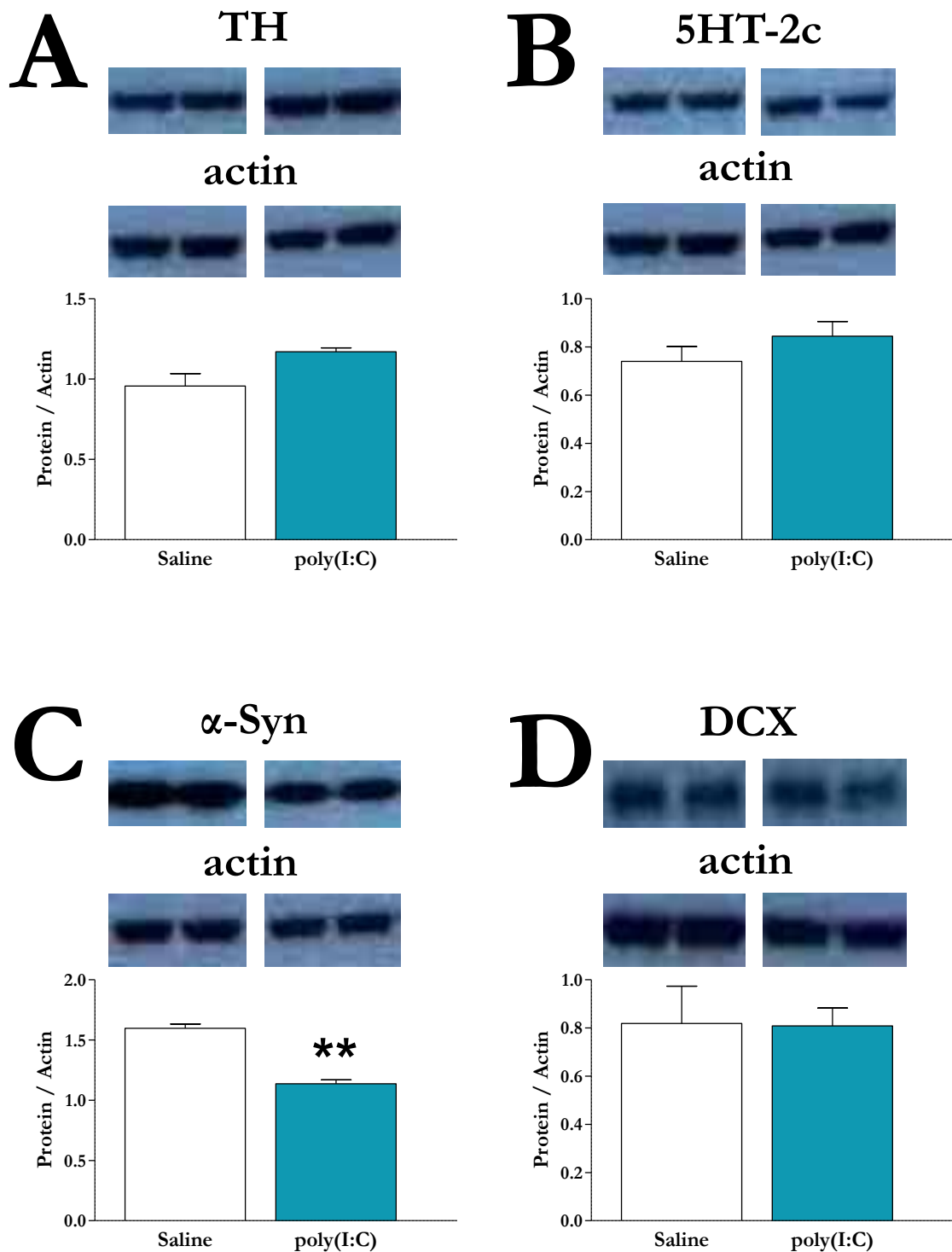


**Figure 3-6. GluN1, DISC-1, RhoA and RhoB expression in poly(I:C)-treated neonatal brains.** Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1, (C) RhoA and (D) RhoB in the brains of rat offspring following maternal exposure to 0.9 % saline or 10 mg/kg poly(I:C) on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 5-6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with poly(I:C). Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



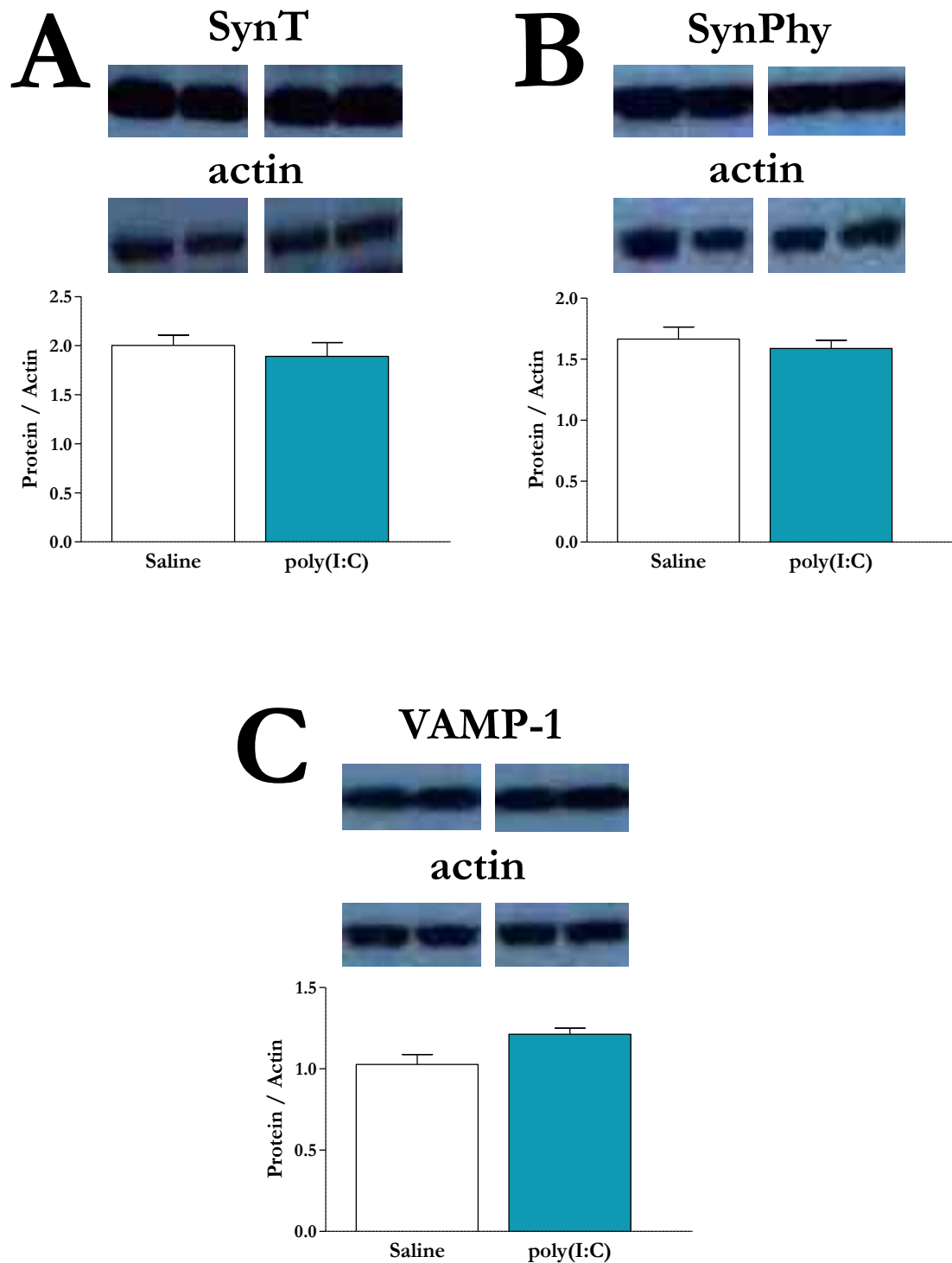
**Figure 3-7. Unc5H1, Unc5H3 and DCC expression in poly(I:C)-treated neonatal brains.**

Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC in the brains of rat offspring following maternal exposure to 0.9 % saline or 10 mg/kg poly(I:C) on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with poly(I:C). Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-8. TH, 5HT-2c, α-Syn and DCX expression in poly(I:C)-treated neonatal brains.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2c, (C) A-Syn and (D) DCX in the brains of rat offspring following maternal exposure to 0.9 % saline or 10 mg/kg poly(I:C) on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with poly(I:C). Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-9. Expression of synaptic proteins in poly(I:C)-treated neonatal brains.**

Bar charts illustrate the quantified protein expression of (A) SynT, (B) SynPhy and (C) VAMP-1 in the brains of rat offspring following maternal exposure to 0.9 % saline or 10 mg/kg poly(I:C) on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 5-6$ , except for VAMP-1 where  $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with poly(I:C). Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



A summary of all changes in protein expression following poly(I:C) treatment in embryo brains and P21 animals can be seen in table 3-1.

**Table 3-1. Summary of protein changes following poly(I:C) treatment.**

<b>Protein Marker</b>	<b>Embryo E18 5h</b>	<b>Postnatal Day 21</b>
GluN1	x	p=<0.01** (dec. in poly(I:C))
DISC-1	x	x
RhoA	x	x
RhoB	x	x
Unc5H1	x	x
Unc5H3	x	x
DCC	x	x
TH	x	x
5HT-2 <sub>c</sub>	x	x
A-Syn	x	p=<0.01** (dec. in poly(I:C))
DCX	x	x
VAMP-1	x	x
SynT	-	x
SynPhy	-	x

### 3.3 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats induces significant delayed alterations of neurodevelopmental proteins in postnatal animals, associated with axonal guidance, neurogenesis and schizophrenia

Since immune activation is accompanied by increased activity of the kynurenine pathway with the induction of IDO, subsequently elevating neurotoxic metabolite concentrations in the CNS (Saito *et al.*, 1992), it was hypothesised that induction of the kynurenine pathway during maternal immune activation may partly be responsible for the emergence of psychotic-like symptoms during adulthood. Since the kynurenine pathway is of considerable interest as a drug target for treatment of various hyperexcitability disorders (Vamos *et al.*, 2009) aimed at reducing neurotoxic kynurenines while promoting the neuroprotectant kynurenic acid (KYNA) attenuating excitotoxic damage, it is predicted that such a mechanism during maternal infection may offer significant protection to the developing offspring potentially lowering the risk of schizophrenia. This suggests alterations in kynurenines during development may provide a convergent intracellular pathway whereby environmental factors, including infection and stress, may exert neurodevelopmental damage. Therefore, the effects of modifying the normal physiological activity of the kynurenine pathway during prenatal development may be predictive of a subsequent increased risk in schizophrenia developing during adulthood. Since cellular polarity and tissue formation may largely be determined in response to a gradient of signal antagonists (Gurdon & Bourillot, 2001) as opposed to an agonist, this would therefore support the concept of a developmental role for KYNA. In order to test this hypothesis, and to evaluate the role of the kynurenine pathway on brain development, timed pregnant rats were administered an intraperitoneal injection of 100 mg/kg Ro61-8048, a selective kynurenine monooxygenase (KMO) inhibitor, on gestation day E18 and embryo brains were collected 5 h later. We sought to examine any immediate effects of Ro61-8048 on protein expression in the embryo brains as a direct consequence to changes in levels of kynurenines during development.

*3.3.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats does not alter the expression of the selected proteins in the embryo brains at 5 h*

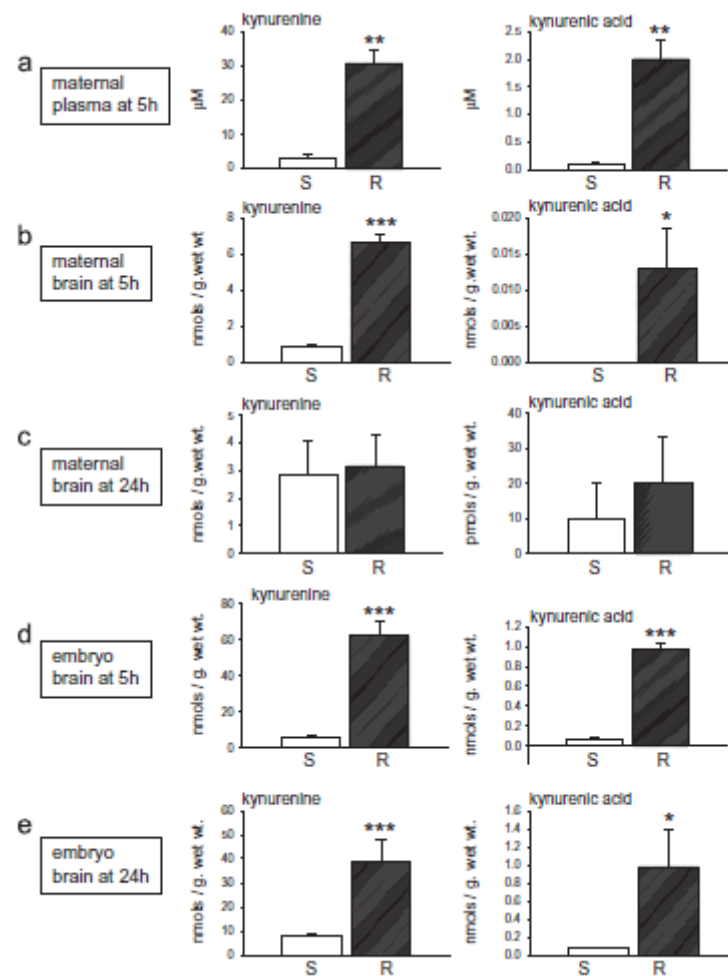
Ro61-8048 treatment in the pregnant mother increases the levels of kynurenine and kynurenine acid in the mother and embryos, confirming the efficacy of the selected dose and elevation of central kynurenines. Kynurenic acid was significantly elevated in the maternal plasma and brain at 5 h, including in the embryo brains at 5 and 24 h (figure 3-10)

The quantified protein expression from western blots showed no change in the brains of embryos 5 h post-treatment with Ro61-8048 in the expression of GluN1, DISC-1, RhoA and RhoB ( $p \geq 0.05$ , figure 3-11).

When the netrin family of proteins were examined in embryo brains, the quantified protein expression also showed no changes in Unc5H1, Unc5H3 and DCC ( $p \geq 0.05$ ) following Ro61-8048 treatment, as seen in figure 3-12.

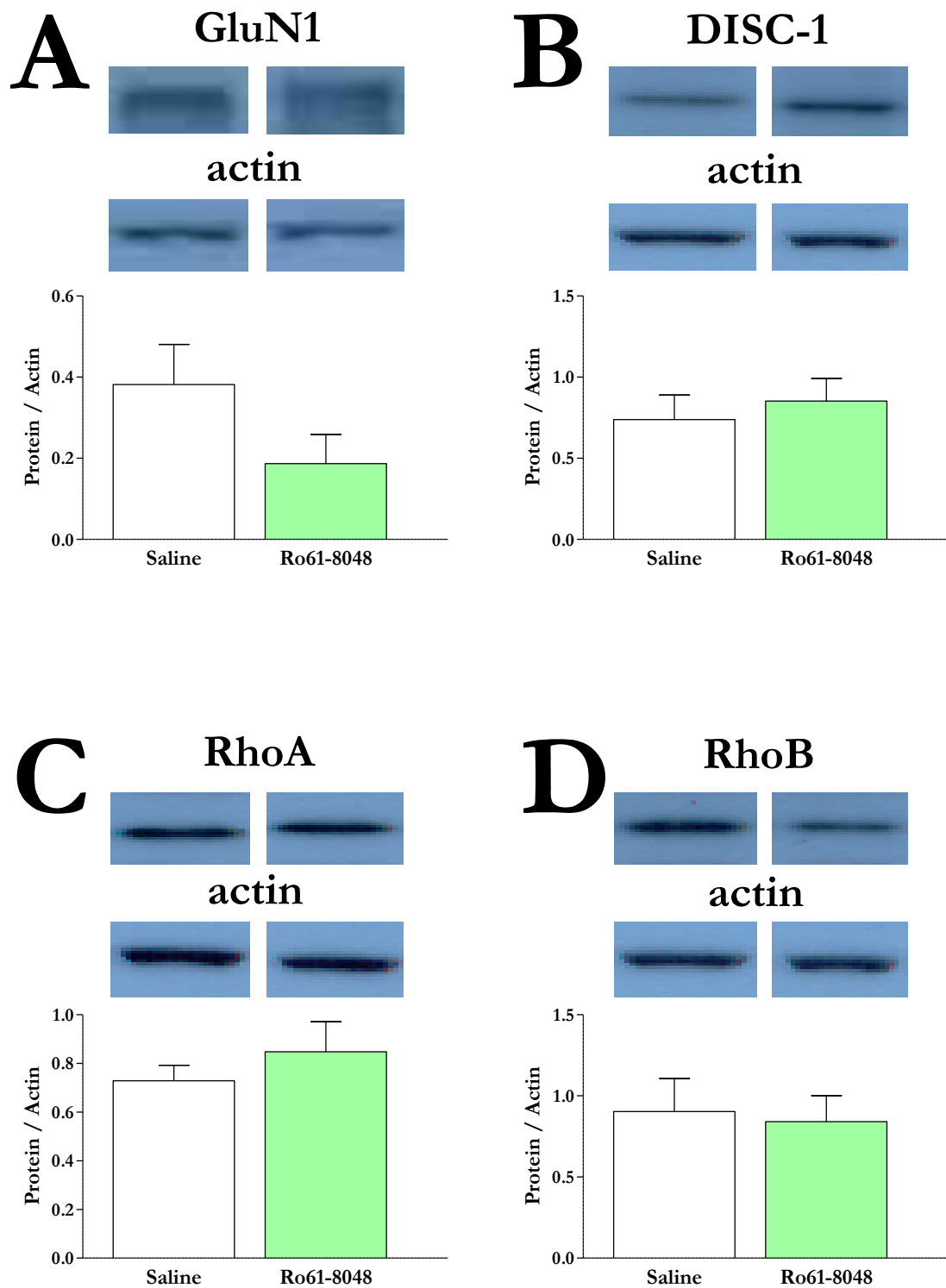
Similarly, the quantified protein expression of tyrosine hydroxylase (TH), serotonin 5HT-2<sub>C</sub> receptor (5HT-2<sub>C</sub>) and  $\alpha$ -synuclein ( $\alpha$ -Syn) also revealed no changes in the embryos at 5 h ( $p \geq 0.05$ , figure 3-13).

Since synaptic markers are present in great abundance during development, in the acute experiments only VAMP-1 (synaptobrevin) was examined to find there was no change in protein expression between the treatment groups ( $p \geq 0.05$ ), while the neurogenesis marker doublecortin (DCX) also showed no significant change following maternal treatment with Ro61-8048 ( $p \geq 0.05$ , figure 3-14).

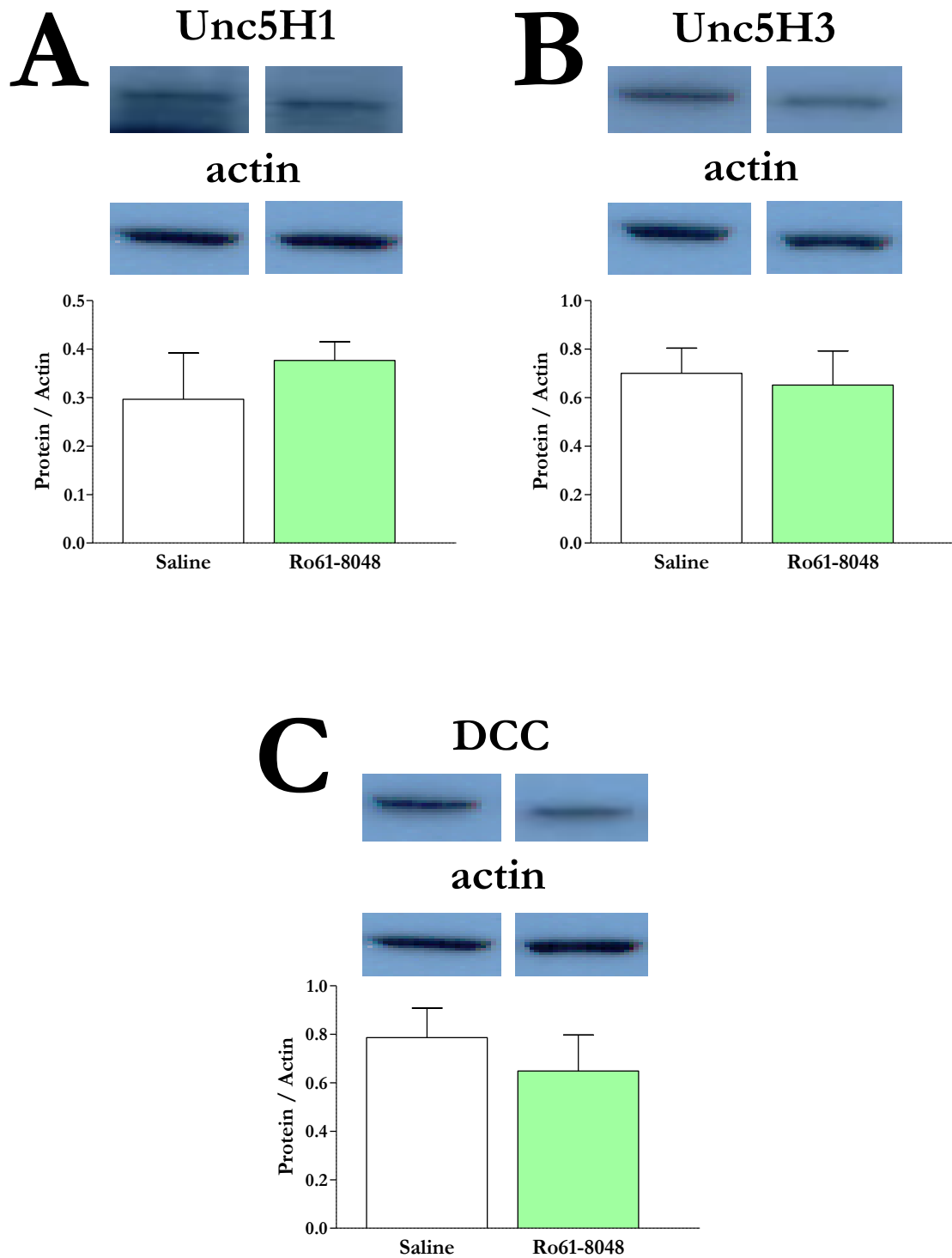


**Figure 3-10. Ro61-8048 administered to the pregnant mother induces changes in kynurenines.**

Bar charts illustrate the concentration of kynurenine and kynurenic acid in (A) maternal plasma at 5 h, (B) maternal brain at 5 h, (C) maternal brain at 24 h, (D) embryo brain at 5 h and (E) embryo brain at 24 h. Results are displayed as  $\pm$  s.e.mean ( $n = 3$ ). Statistical comparisons were made using an unpaired  $t$  test between groups of animals treated with saline and groups of animals treated with Ro61-8048. A probability value of 0.05 was set as the criterion for significance.

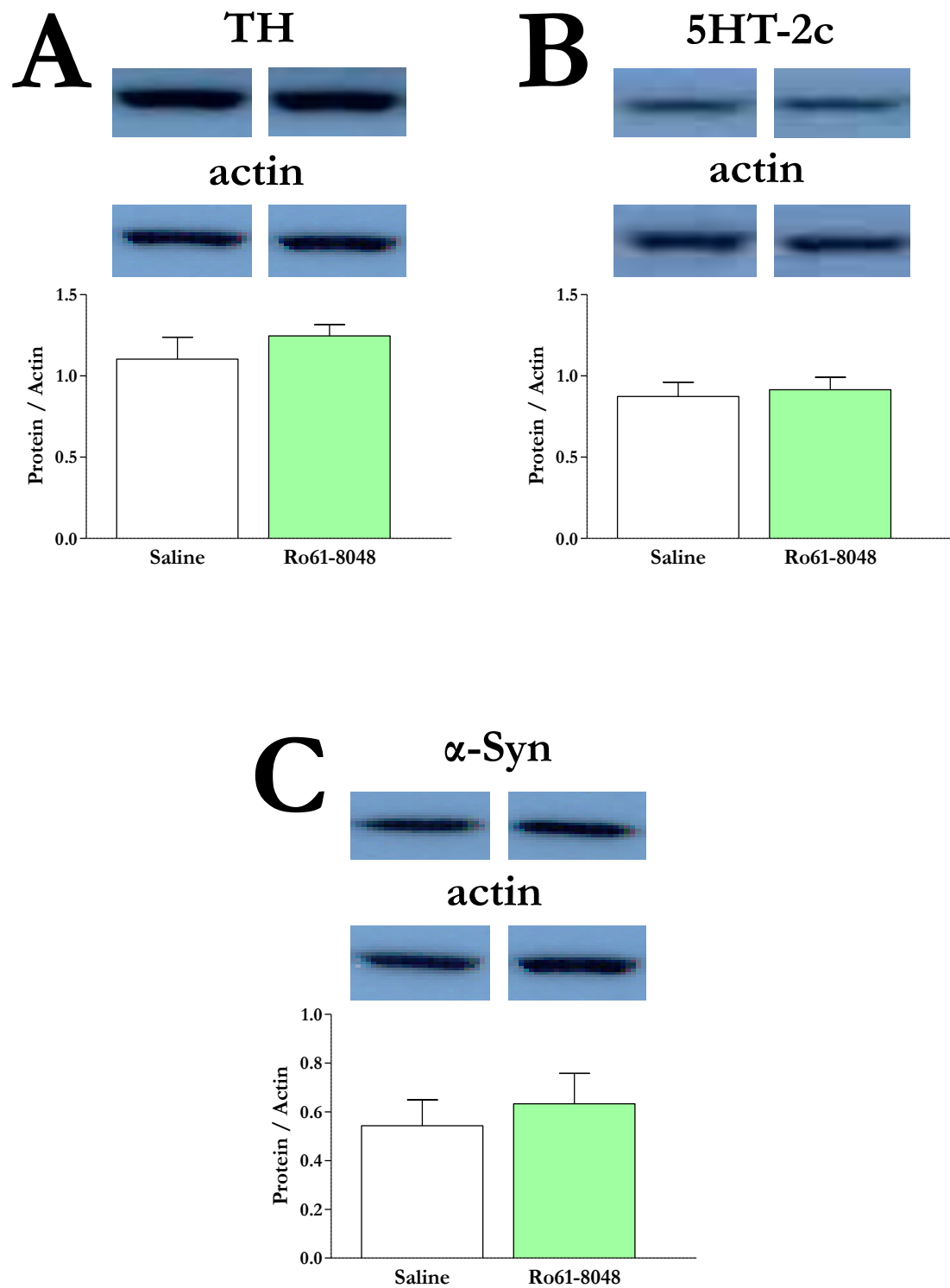


**Figure 3-11. GluN1, DISC-1, RhoA and RhoB expression in Ro61-8048-treated embryo brains.** Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1, (C) RhoA and (D) RhoB in the brains of the embryo following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation day E18. Pregnant mothers were euthanized 5 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



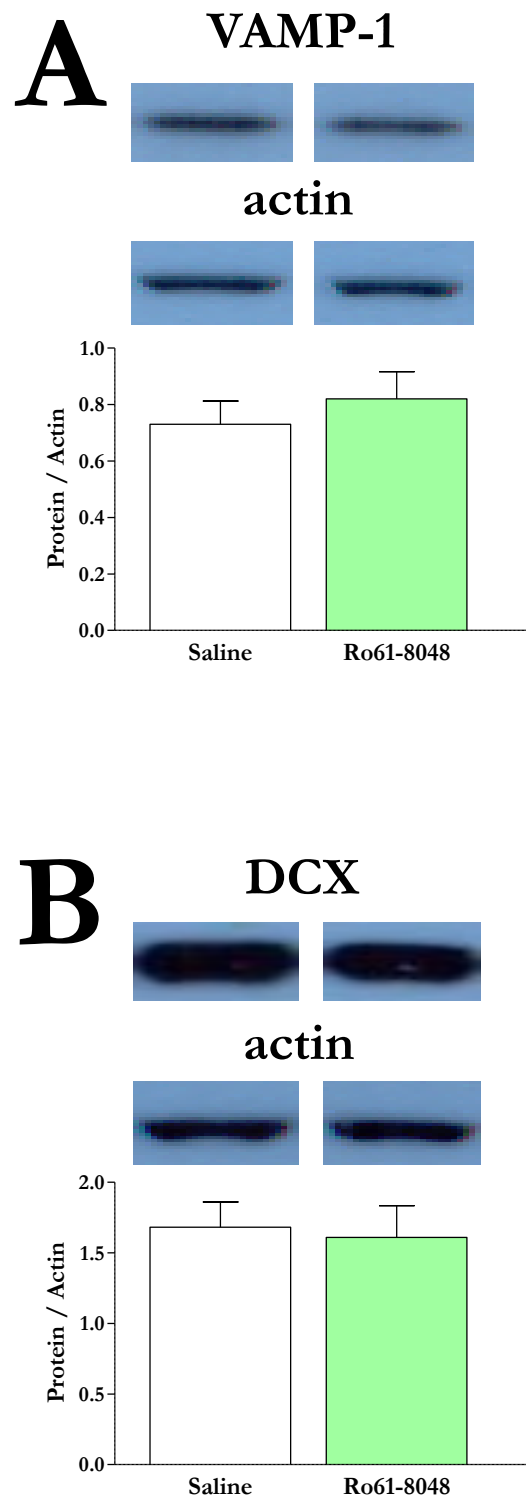
**Figure 3-12. Unc5H1, Unc5H3 and DCC expression in Ro61-8048-treated embryo brains.**

Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC in the brains of the embryo following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation day E18. Pregnant mothers were euthanized 5 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-13. TH, 5HT-2c and  $\alpha$ -synuclein expression in Ro61-8048-treated embryo brains.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2c and (C) A-Syn in the brains of the embryo following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation day E18. Pregnant mothers were euthanized 5 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-14. VAMP-1 and doublecortin expression in Ro61-8048-treated embryo brains.**

Bar charts illustrate the quantified protein expression of (A) VAMP-1 and (B) DCX in the brains of the embryo following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation day E18. Pregnant mothers were euthanized 5 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



### 3.4 Examination of Protein Markers in Embryo Brains at 24 h

Since Ro61-8048 produced no acute changes in the embryos at 5 h post-treatment, this suggests Ro61-8048 may induce a more delayed response. This may be due to time requirements needed for the homeostatic shift in kynurenine levels and to allow time for subsequent changes to occur. Therefore, to address this issue, timed pregnant rats were administered an intraperitoneal injection of 100 mg/kg Ro61-8048 on E18 and embryo brains were collected at 24 h post-injection at E19, and any changes in protein expression were examined in brain lysates.

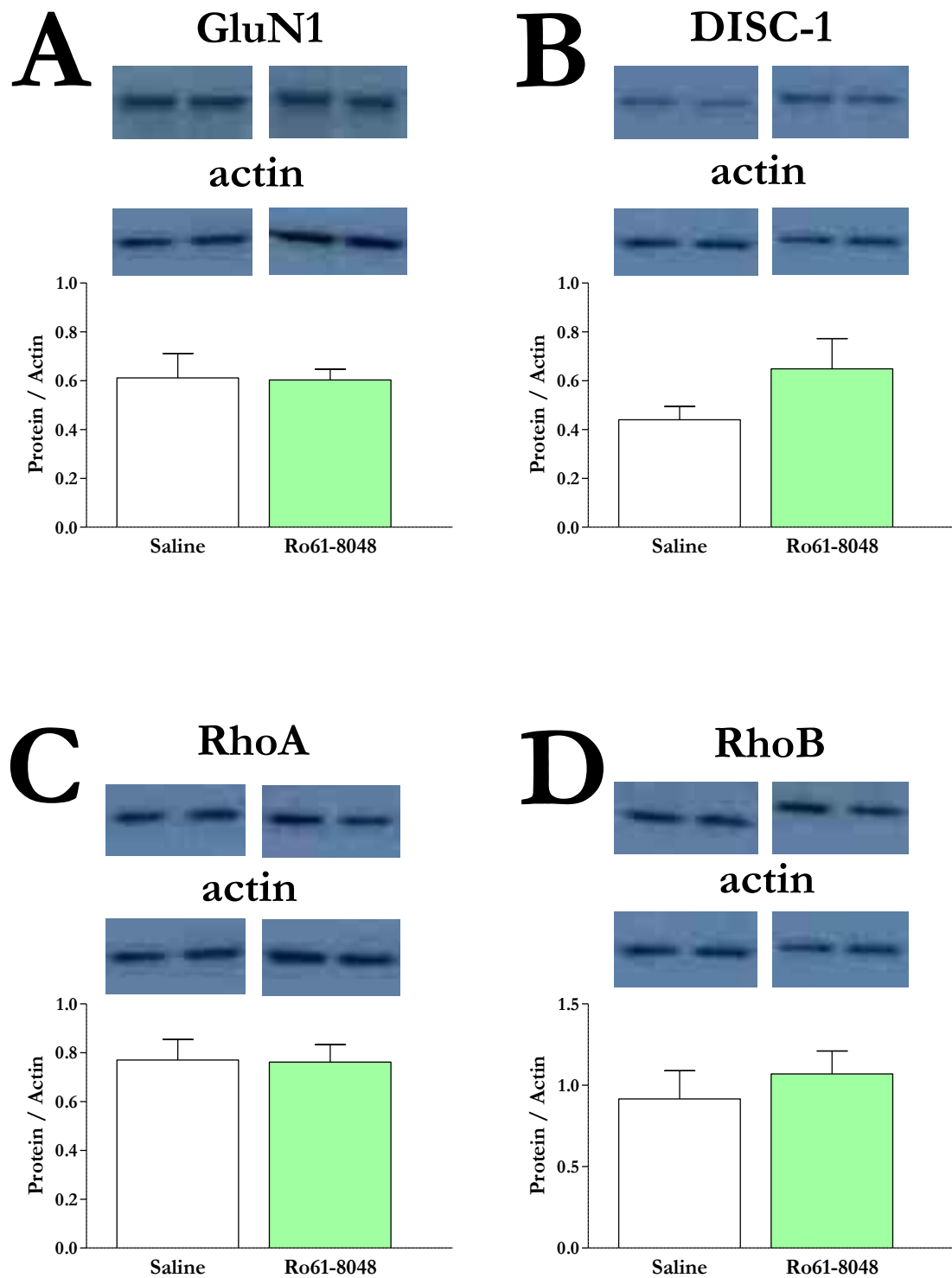
*3.4.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats does not alter the expression of the selected proteins in the embryo brains at 24 h*

The quantified protein expression from western blots showed no changes in the embryo brains at 24 h post-treatment with Ro61-8048 in GluN1, DISC-1, RhoA and RhoB ( $p > 0.05$ , figure 3-15).

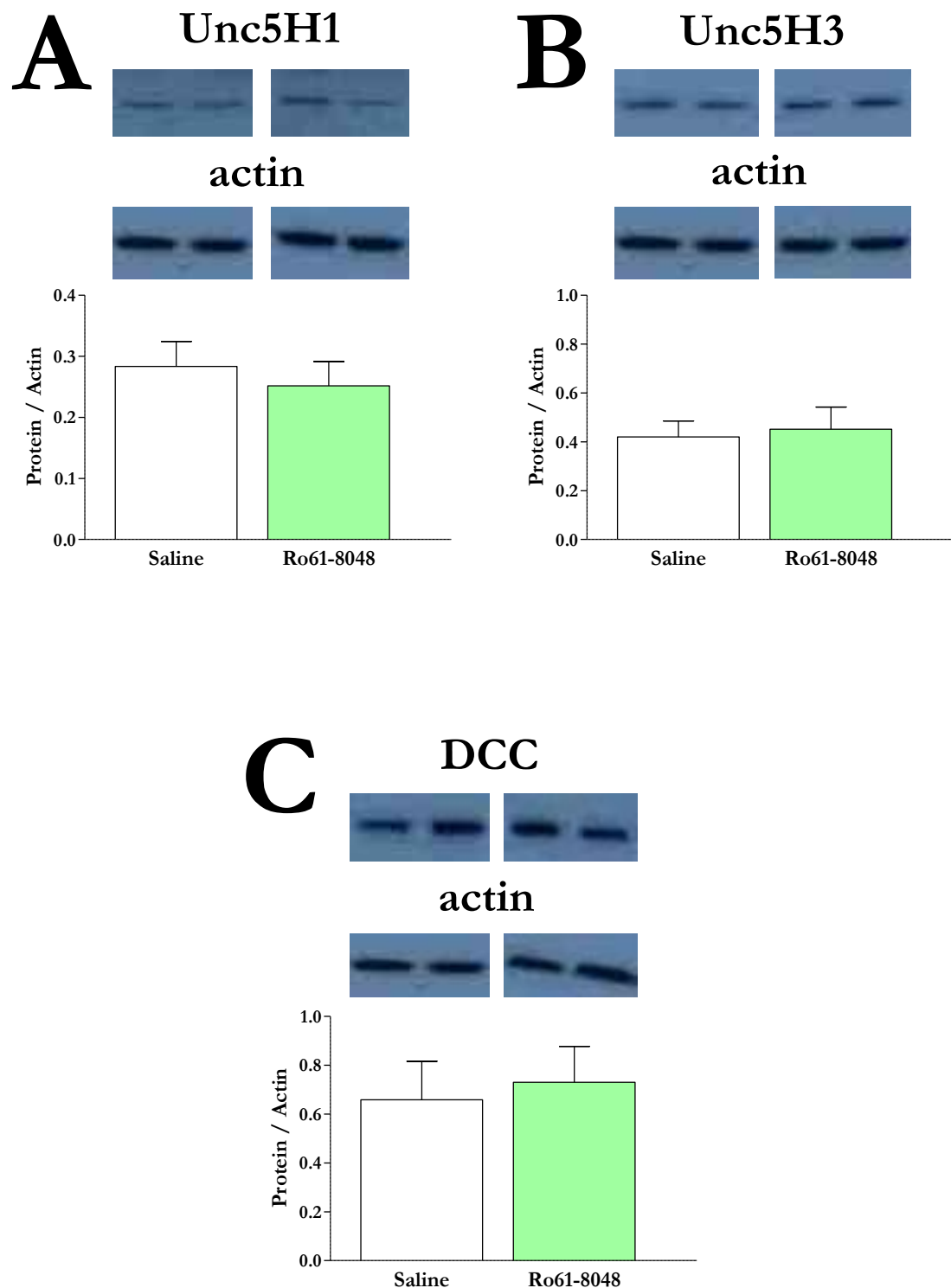
When the netrin family of proteins were examined, the quantified protein expression from western blots showed no changes in Unc5H1, Unc5H3 and DCC ( $p > 0.05$ ) following Ro61-8048 treatment (figure 3-16).

Similarly, the protein expression of tyrosine hydroxylase (TH), serotonin 5HT-2<sub>C</sub> receptors (5HT-2<sub>C</sub>) and  $\alpha$ -synuclein also remained unchanged ( $\alpha$ -Syn ;  $p > 0.05$ , figure 3-17).

Since synaptic markers are present in great abundance during development, in the acute experiments, only VAMP-1 (synaptobrevin) was examined to find no change in protein expression between treatment groups ( $p > 0.05$ ), while the neurogenesis marker doublecortin (DCX) also showed no change following maternal treatment with Ro61-8048 ( $p > 0.05$ , figure 3-18).

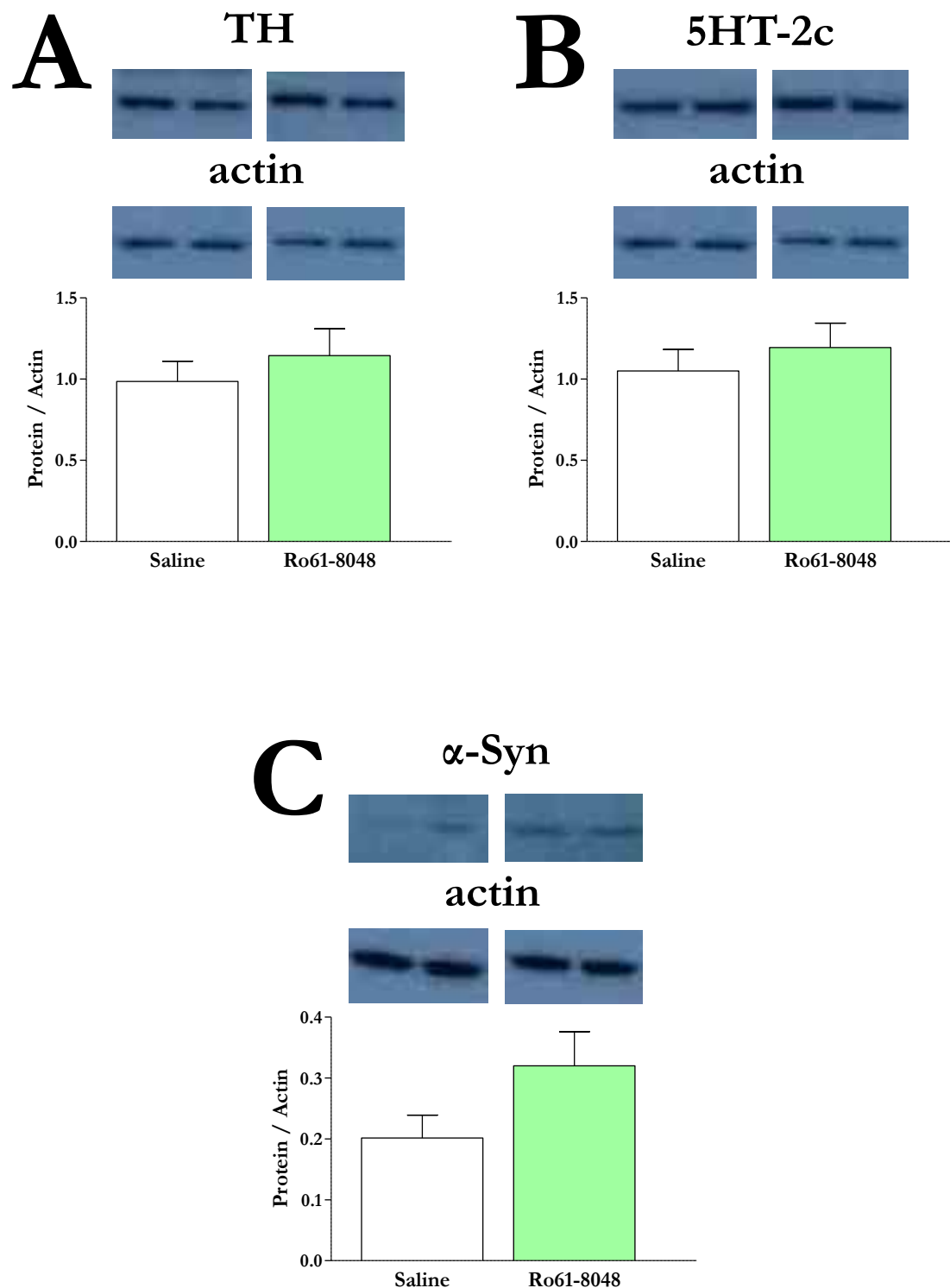


**Figure 3-15. GluN1, DISC-1, RhoA and RhoB expression in Ro61-8048-treated embryo brains.** Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1, (C) RhoA and (D) RhoB in the brains of the embryo following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation day E18. Pregnant mothers were euthanized 24 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



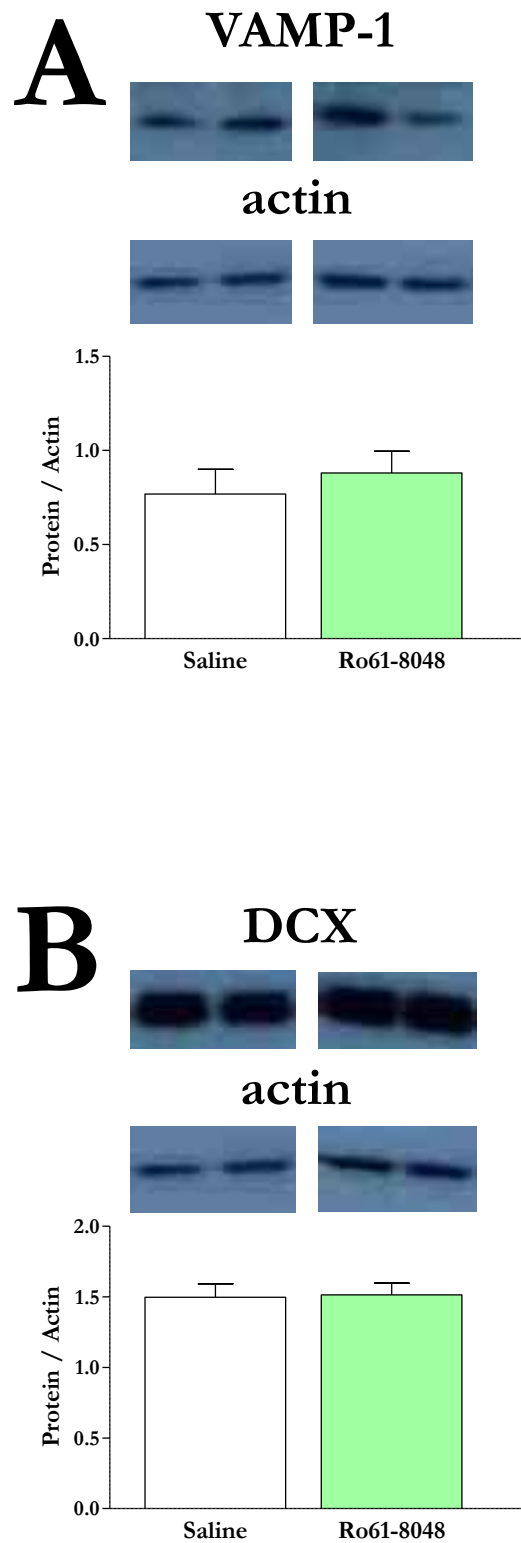
**Figure 3-16. Unc5H1, Unc5H3 and DCC expression in Ro61-8048-treated embryo brains.**

Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC in the brains of the embryo following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation day E18. Pregnant mothers were euthanized 24 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-17. TH, 5HT-2c and  $\alpha$ -synuclein expression in Ro61-8048-treated embryo brains.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2c and (C) A-Syn in the brains of the embryo following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation day E18. Pregnant mothers were euthanized 24 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-18. VAMP-1 and doublecortin expression in Ro61-8048-treated embryo brains.**

Bar charts illustrate the quantified protein expression of (A) VAMP-1 and (B) DCX in the brains of the embryo following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation day E18. Pregnant mothers were euthanized 24 h post-injection and embryo brains analysed by western blotting for any relative or absolute changes in protein expression. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of embryos whose mothers were treated with saline and groups of embryos whose mothers were treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.

### 3.5 Examination of Protein Markers in P21 Animals

Since Ro61-8048 failed to induce significant alterations in the acute experiments, postnatal changes were examined in animals at 21 days of age. Since schizophrenia symptoms usually exacerbate with age progression until adulthood (Weinberger, 1987), it was expected that more profound changes may be seen in postnatal animals. Therefore, we sought to examine whether inhibiting the kynurenine pathway prenatally could induce delayed changes in brain development. To this end, timed pregnant rats were administered an intraperitoneal injection of 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. A triple injection schedule was selected to maximise the duration the kynurenine pathway was disrupted, and to examine its subsequent effects upon brain development. Brains of P21 animals were removed and dissected separating the two cerebral hemispheres and processed for western blotting. While the examination of whole half brain lysates could limit the effectiveness of identifying changes in protein expression, as subtle changes in particular markers such as tyrosine hydroxylase and  $\alpha$ -Synuclein may be more relevant to more localised regions like the striatum and substantia nigra, since these markers are also abundantly expressed in many other regions of the brain, including  $\alpha$ -synuclein being a synaptic protein, it could be that examining the protein expression in limited areas of the striatum would prevent the identification of an overall change. Furthermore, the majority of markers evaluated serve predominant roles in neurite development, axonal growth, neuronal migration, synaptogenesis and transmitter release, largely relevant to neuronal process. Since these proteins are located throughout the brain, we sought to examine if Ro61-8048 could induce overall gross changes in these markers to alter overall brain function.

*3.5.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats alters the expression of proteins associated with the function of NMDA receptors in P21 brains*

When protein expression was compared in postnatal animals, GluN1, DISC-1 and Post-Synaptic Density (PSD)-95 levels were comparable with control values ( $p \geq 0.05$ ), as seen in figure 3-19.

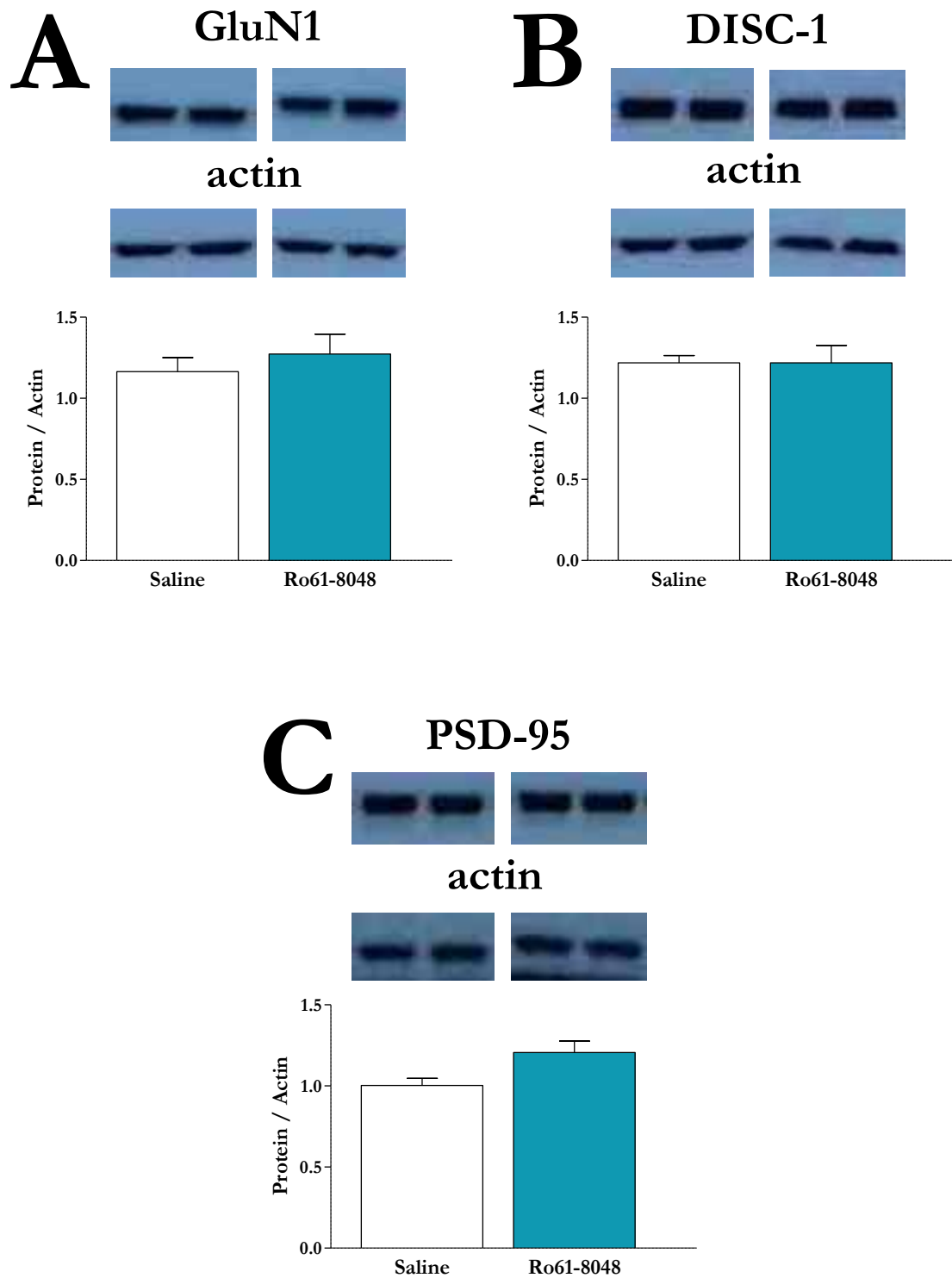
While Ro61-8048 produced no change in the overall protein content of RhoA ( $p \geq 0.05$ ), interestingly, there was a selective decrease in the protein expression of RhoB ( $p < 0.05^*$ , figure 3-20).

No significant changes were seen in the netrins including: Unc5H1, Unc5H3 and DCC ( $p \geq 0.05$ ) in P21 animals as seen in figure 3-21.

Similarly at P21, the levels of doublecortin (DCX), tyrosine hydroxylase (TH), 5HT-2c and  $\alpha$ -synuclein ( $\alpha$ -Syn) remained comparable with controls ( $p \geq 0.05$ ), as seen in figure 3-22.

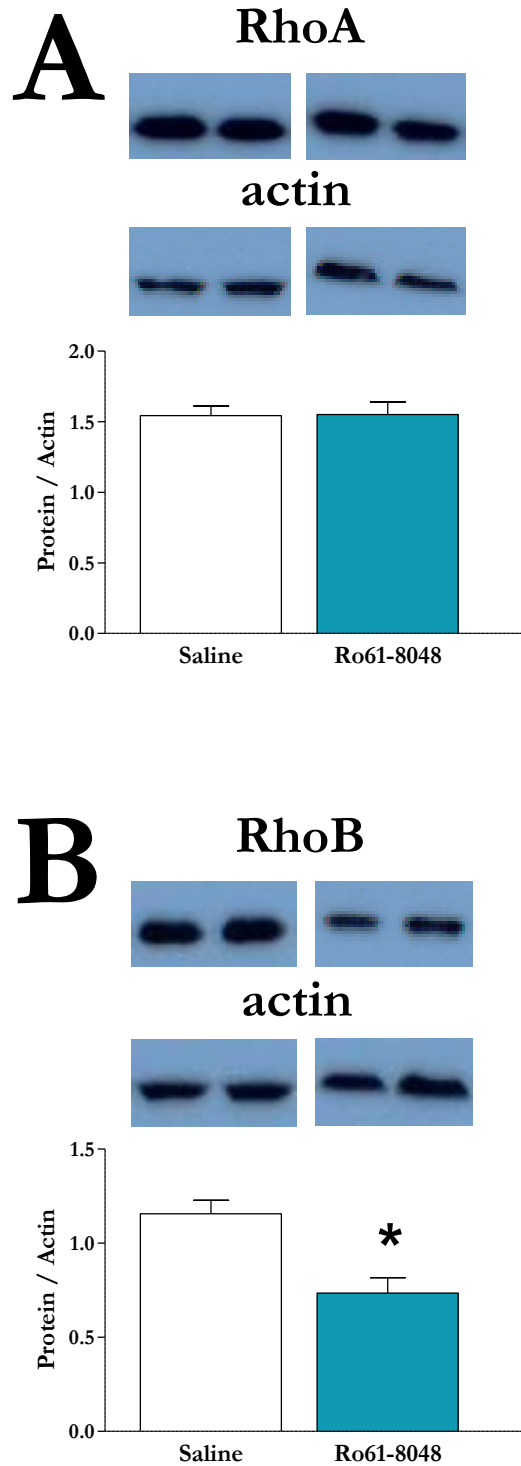
When the protein expression of VAMP-1 (synaptobrevin) was assessed, its expression remained comparable with controls ( $p \geq 0.05$ ). Protein expression of synaptic markers synaptotagmin (SynT) and synaptophysin (SynPhy) were also examined to find no significant difference existed between treatment groups ( $p \geq 0.05$ ) as seen in figure 3-23.





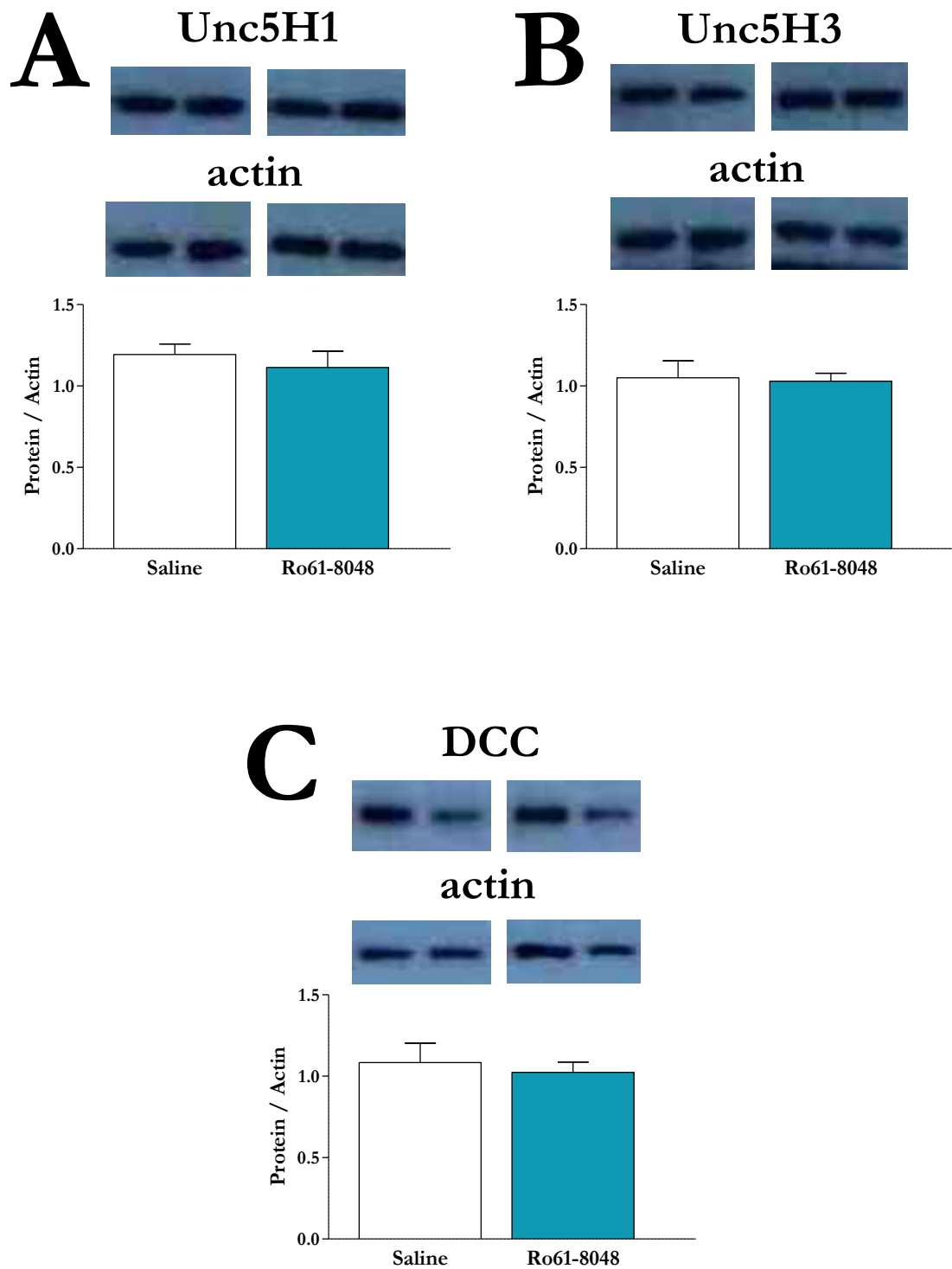
**Figure 3-19. GluN1, DISC-1 and PSD-95 expression in Ro61-8048-treated neonatal brains.**

Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1 and (C) PSD-95 in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



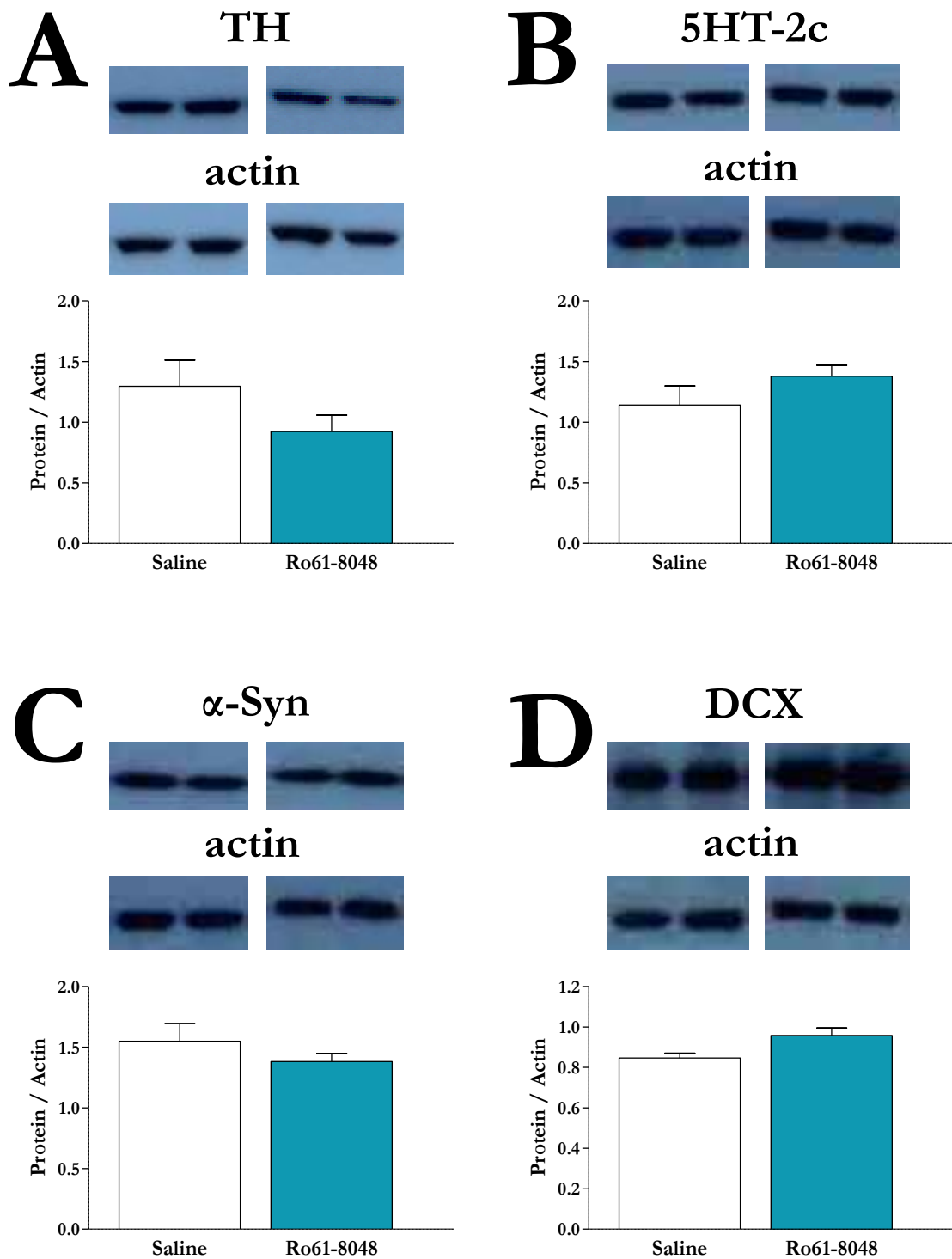
**Figure 3-20. RhoA and RhoB expression in Ro61-8048-treated neonatal brains.**

Bar charts illustrate the quantified protein expression of (A) RhoA and (B) RhoB in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



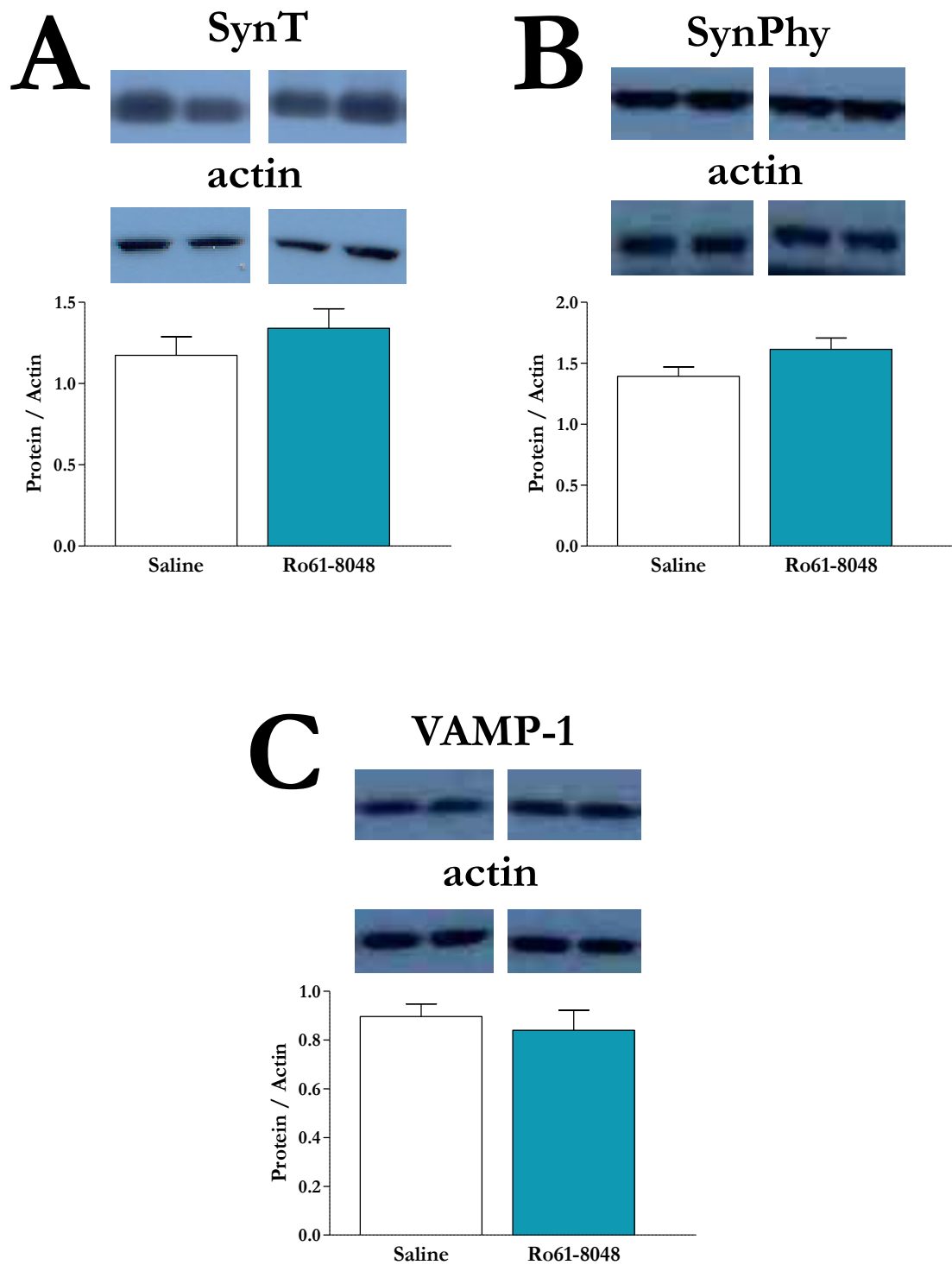
**Figure 3-21. Unc5H1, Unc5H3 and DCC expression in Ro61-8048-treated neonatal brains.**

Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-22. TH, 5HT-2c,  $\alpha$ -Syn and DCX expression in Ro61-8048-treated neonatal brains.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2c, (C) A-Syn and (D) DCX in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n=6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-23. Expression of synaptic proteins in Ro61-8048-treated neonatal brains.**

Bar charts illustrate the quantified protein expression of (A) SynT, (B) SynPhy and (C) VAMP-1 in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Neonates were sacrificed at P21 and their brains removed to examine any relative or absolute changes in protein expression analysed in half of the cerebral hemisphere by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.

### 3.6 Examination of Protein Markers in P60 Animals

While RhoB was affected in P21 animals, demonstrating Ro61-8048 treatment affects brain development, we sought to compare this change in older animals during early adulthood. Firstly, we sought to identify whether this change persisted into adulthood, and secondly, since this is around the time the full blown symptoms for schizophrenia become apparent, it was hypothesised this time period may be associated with more dramatic alterations in protein expression. As prenatal inhibition of KMO with Ro61-8048 demonstrated a clear efficacy in precipitating changes in protein expression, in P60 animals it was attempted to localise where some of these changes may be occurring. While it is possible identifying gross changes in half brain lysates may be compromised as the effects of subtle changes in opposite directions in different brain regions would yield no overall net change in protein expression, or that subtle changes are masked by other regions thereby out-with the limit of detection with the western blots, P60 rat brains were dissected into regions of the hippocampus, cortex, midbrain and cerebellum.

### 3.6.1 Examination of Protein Markers in the P60 Hippocampus

The hippocampus of P60 animals was examined since it is identified as one of the central regions involved in the neuropathology and neurophysiology of schizophrenia. This evidence comes from a range of sources including *in vivo* models showing neurophysiological, structural and functional deficits, including from post-mortem brains of schizophrenia patients demonstrating deficits in histology, morphometry, gene expression and hippocampal neurochemistry (Reviewed in Harrison, 2004). Neuropathological deficits largely illustrate abnormalities in neuronal morphology, while the organisation of presynaptic and dendritic parameters appear to be largely dis-regulated, suggesting altered synaptic neurocircuitry of hippocampal ‘wiring’ in its extrinsic connections. Glutamatergic signalling is prominently affected with a loss in excitatory transmitter receptors and transporters (Harrison *et al.*, 1999; Harrison *et al.*, 2006; Shan *et al.*, 2013), while alteration in GABAergic signalling is also implicated (Heckers, 2002, 2004; Perez and Lodge, 2013). Since the hippocampus is largely responsible for higher cognitive functions including plasticity, learning and memory, the involvement of the hippocampus in schizophrenia is predictive to be associated with neuropsychological impairments as opposed to psychotic symptoms. Interestingly, a large proportion of hippocampal dysfunction, including reductions in hippocampal volume (Johnson *et al.*, 2013), has been attributed to aberrant glutamatergic signalling as a principal driver behind these impairments (Schobel *et al.*, 2013). Therefore, we sought to examine any changes in protein expression of neuronal markers following Ro61-8048 treatment *in utero*, in the hippocampus of P60 animals that may potentially precipitate hippocampal pathology leading to heightened risks of schizophrenia.

3.6.1.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats significantly increases the protein expression of doublecortin (DCX) in the hippocampus of postnatal day 60 animals

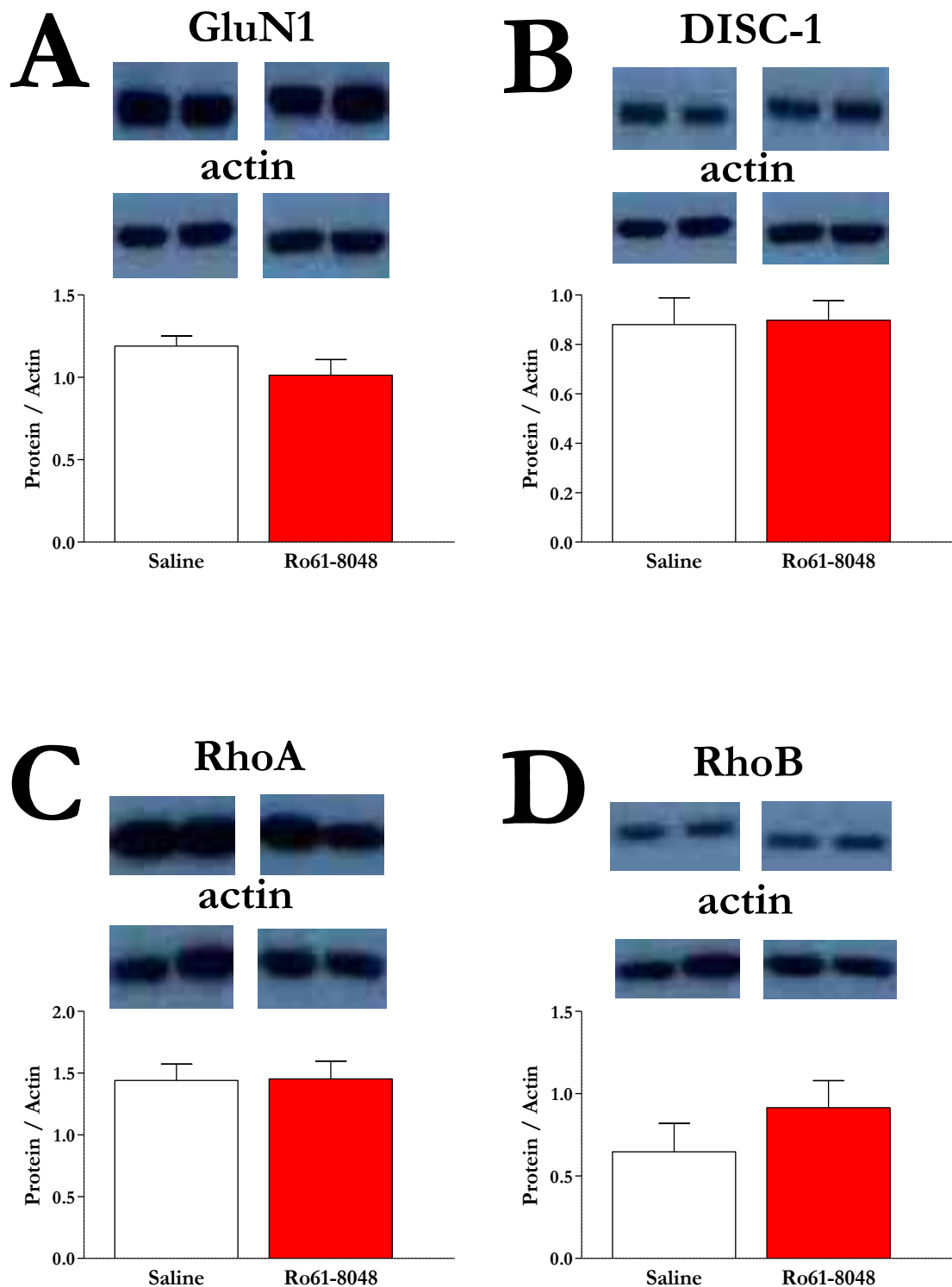
Examination of the hippocampus by immunoblotting in P60 animals revealed no changes in the protein expression of GluN1, DISC-1, RhoA and RhoB ( $p \geq 0.05$ , figure 3-24).

Similarly, no significant changes were seen in the netrin proteins: Unc5H1, Unc5H3 and DCC ( $p \geq 0.05$ ) in the hippocampus of P60 animals, as seen in figure 3-25.

Interestingly, while the protein expression of tyrosine hydroxylase (TH), 5HT-2c and  $\alpha$ -synuclein ( $\alpha$ -Syn) remained comparable with controls ( $p \geq 0.05$ ), doublecortin (DCX) was significantly elevated in groups of animals born to Ro61-8048-treated mothers ( $p < 0.05^*$ ), as seen in figure 3-26.

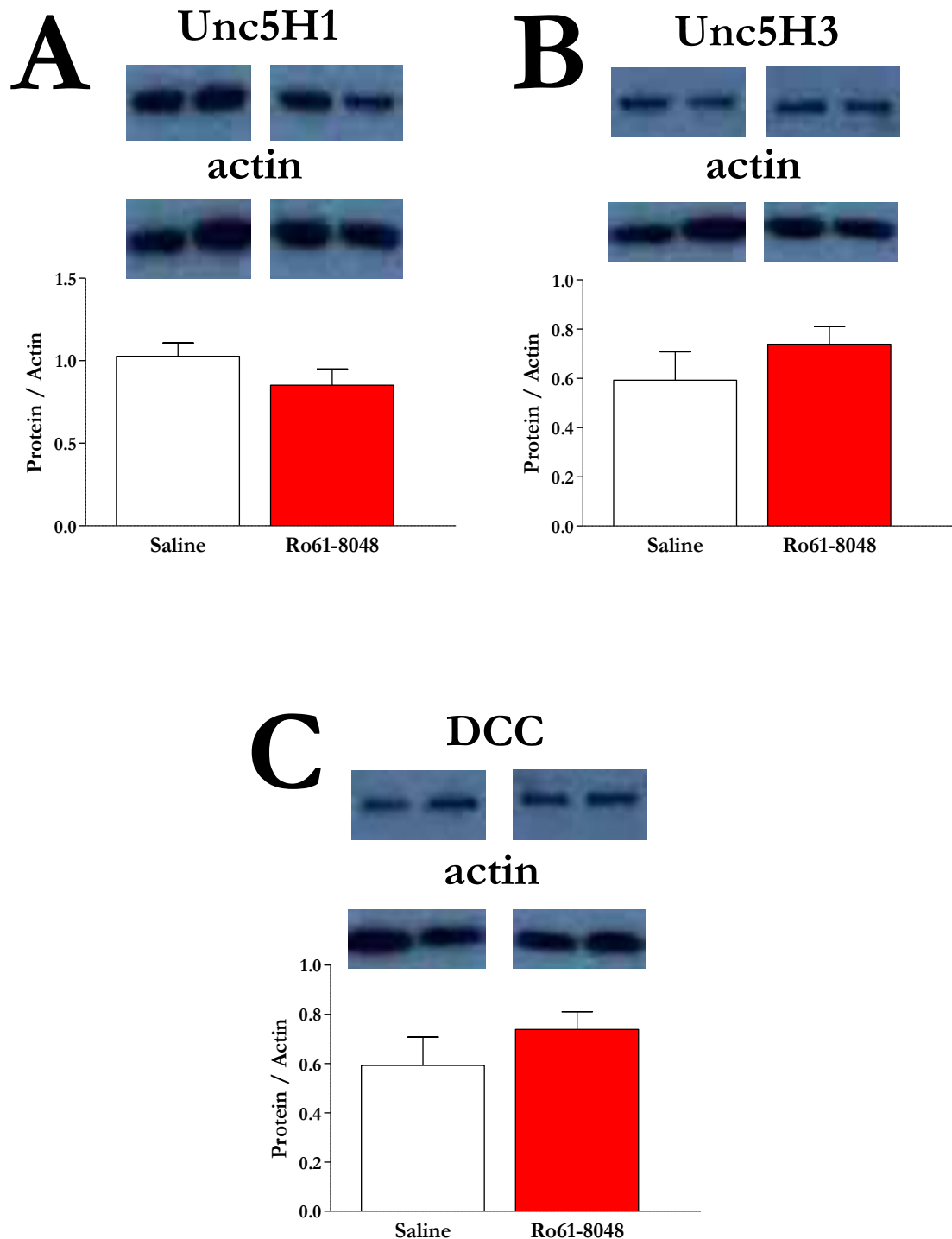
When the protein expression of VAMP-1 (synaptobrevin) was assessed in the hippocampus, its expression remained comparable with controls ( $p \geq 0.05$ ). Synaptic markers synaptotagmin (SynT) and synaptophysin (SynPhy) were also examined to find no significant difference existed between treatment groups ( $p \geq 0.05$ ) as seen in figure 3-27.





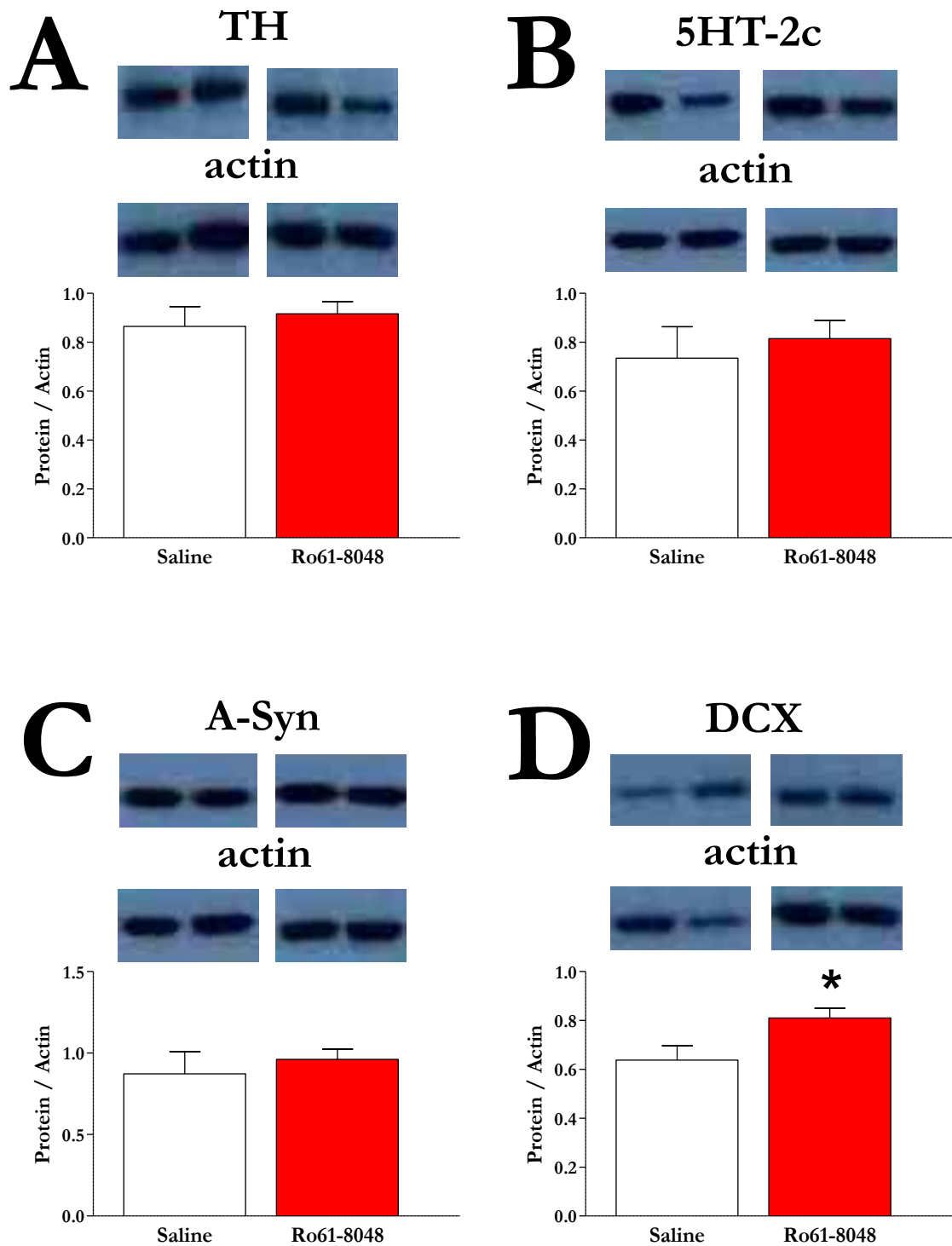
**Figure 3-24. GluN1, DISC-1, RhoA and RhoB expression in the P60 hippocampus.**

Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1, (C) RhoA and (D) RhoB in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the hippocampus by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 4$  for saline,  $n = 6$  for Ro61-8048) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



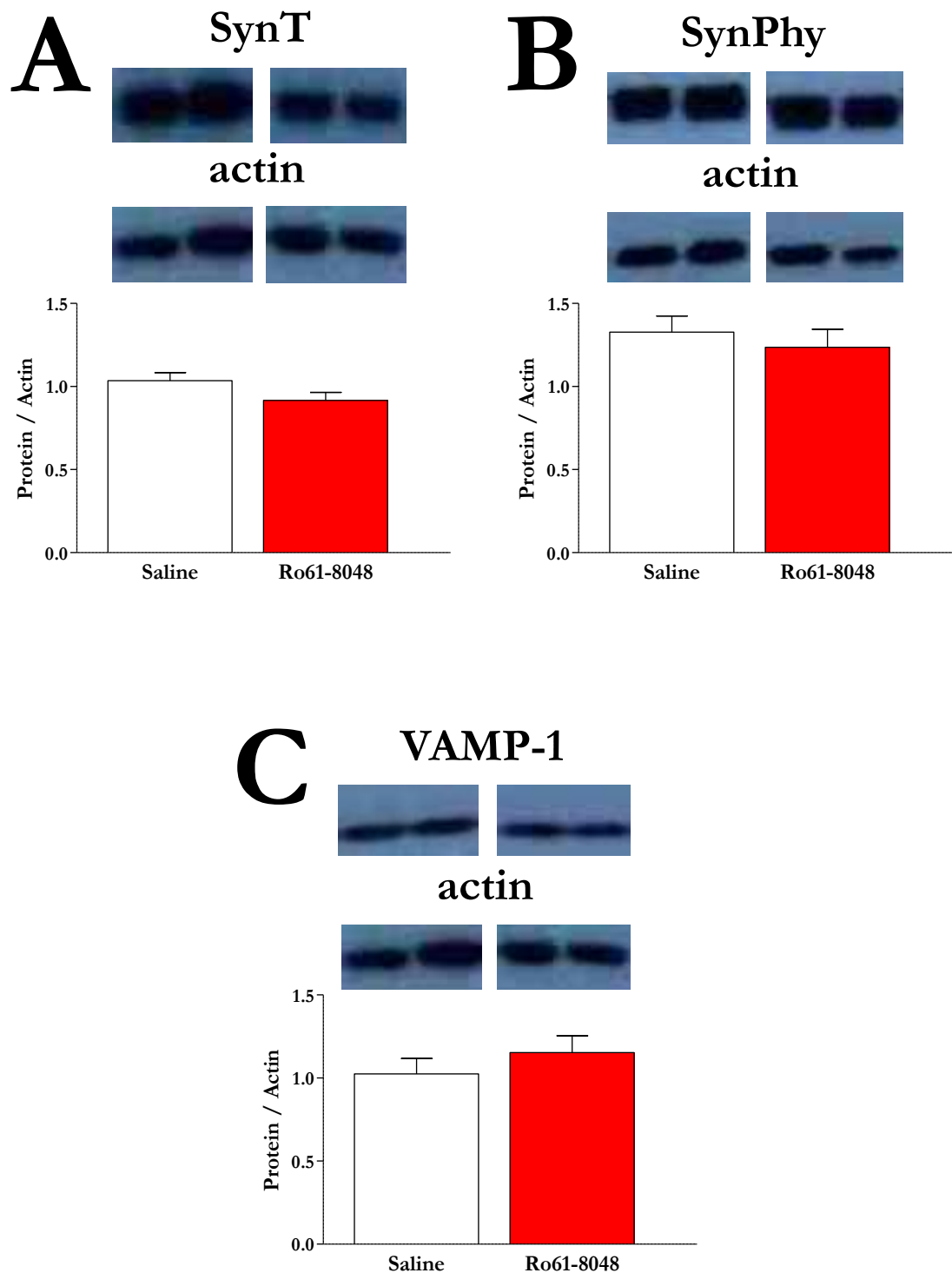
**Figure 3-25. Unc5H1, Unc5H3 and DCC expression in the P60 hippocampus.**

Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the hippocampus by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 4$  for saline,  $n = 6$  for Ro61-8048) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-26. TH, 5HT-2c,  $\alpha$ -synuclein and DCX expression in the P60 hippocampus.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2c, (C) A-Syn and (D) DCX in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the hippocampus by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 4$  for saline,  $n = 6$  for Ro61-8048) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-27. Expression of synaptic proteins in the P60 hippocampus.**

Bar charts illustrate the quantified protein expression of (A) SynT, (B) SynPhy and (C) VAMP-1 in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the hippocampus by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 4$  for saline,  $n = 6$  for Ro61-8048) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.

### 3.6.2 Examination of Protein Markers in the P60 Cortex

Schizophrenia has long been attributed to significant impairments within the cortex. Animal models, post-mortem studies and microarrays reveal significant abnormalities in gene and protein expression of a variety of receptor and transmitter systems including: dopamine, glutamate and GABA (reviewed in Feldon and Folsom, 2009). Specifically, regions of the prefrontal cortex including the prelimbic, infralimbic, orbitofrontal and cingulate cortex appear to be mostly affected. Other cortical areas involved in schizophrenia include the sensory cortex, in addition to cerebellar involvement via cortico-cerebellar tracts involved in precipitating deficits in sensorimotor gating (Teale *et al.*, 2013), while auditory and visual cortices, including the hippocampus, are important for mediating symptoms of hallucinations. Neuroanatomical studies reveal altered cortical organisation of neuronal networks with abnormalities in functional connectivity (Kannan *et al.*, 2009; Chen *et al.*, 2013), thought to underlie deficits in higher cognitive functions including altered patterns of thought processes. Since the frontal cortex is predominantly responsible for cognitive processes, in addition to hippocampal involvement, cortical deficits during schizophrenia mediate significant cognitive abnormalities and reduced intelligence rates (Rusch *et al.*, 2007; Brown *et al.*, 2009; Eriksen *et al.*, 2009). Therefore, alterations in cortical function and development were assessed by examining changes in protein expression following treatment with Ro61-8048.

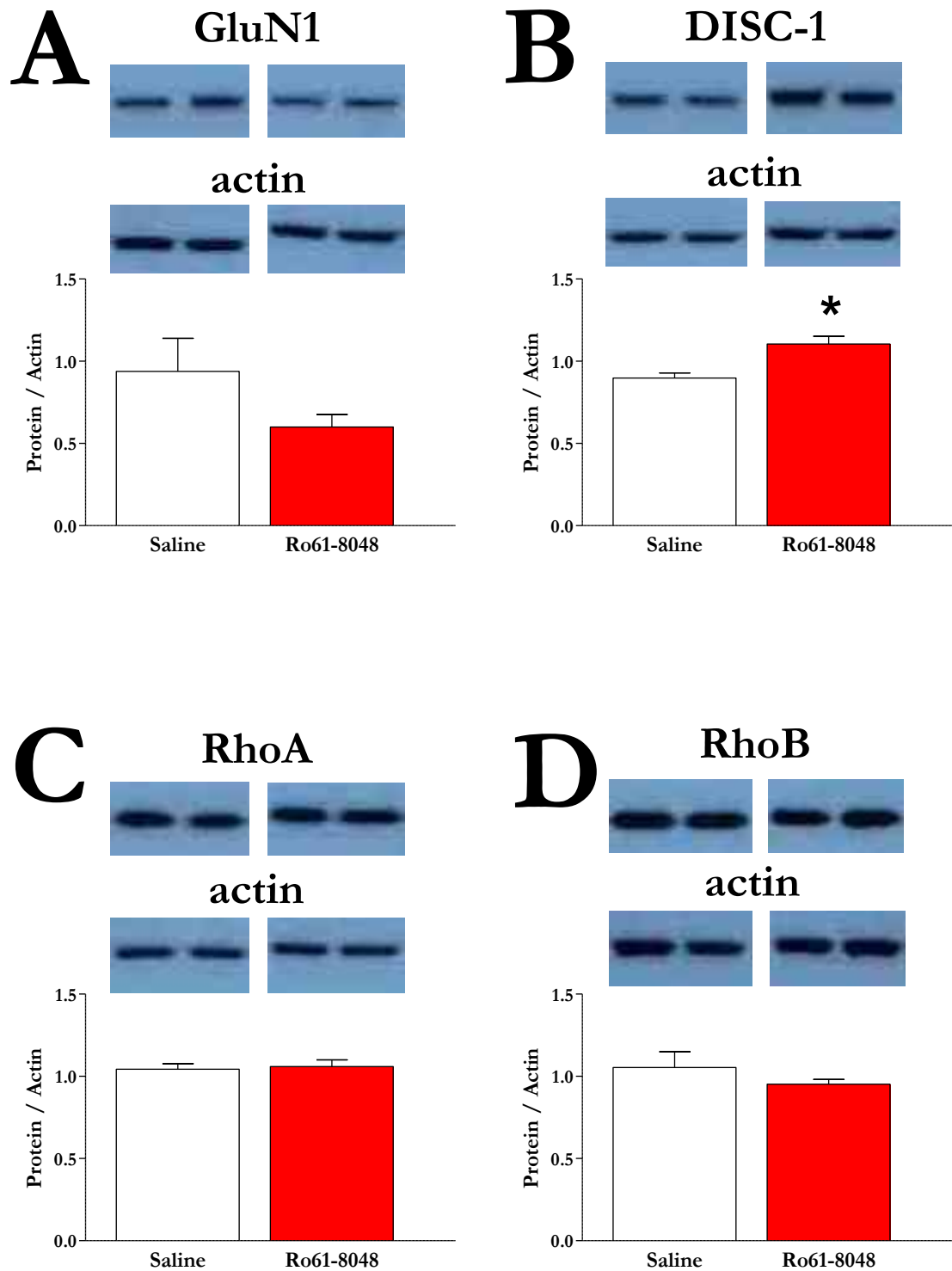
3.6.2.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats significantly increases the protein expression of DISC-1 in the cortex of postnatal day 60 animals

Examination of the cortex by immunoblotting in P60 animals revealed no changes in protein expression of GluN1, RhoA and RhoB ( $p \geq 0.05$ ), while DISC-1 was significantly elevated in groups of animals exposed to Ro61-8048 prenatally, when compared with controls ( $p < 0.05$ \* figure 3-28).

The cortex revealed no significant changes in Unc5H1, Unc5H3 and DCC ( $p \geq 0.05$ ), as seen in figure 3-29.

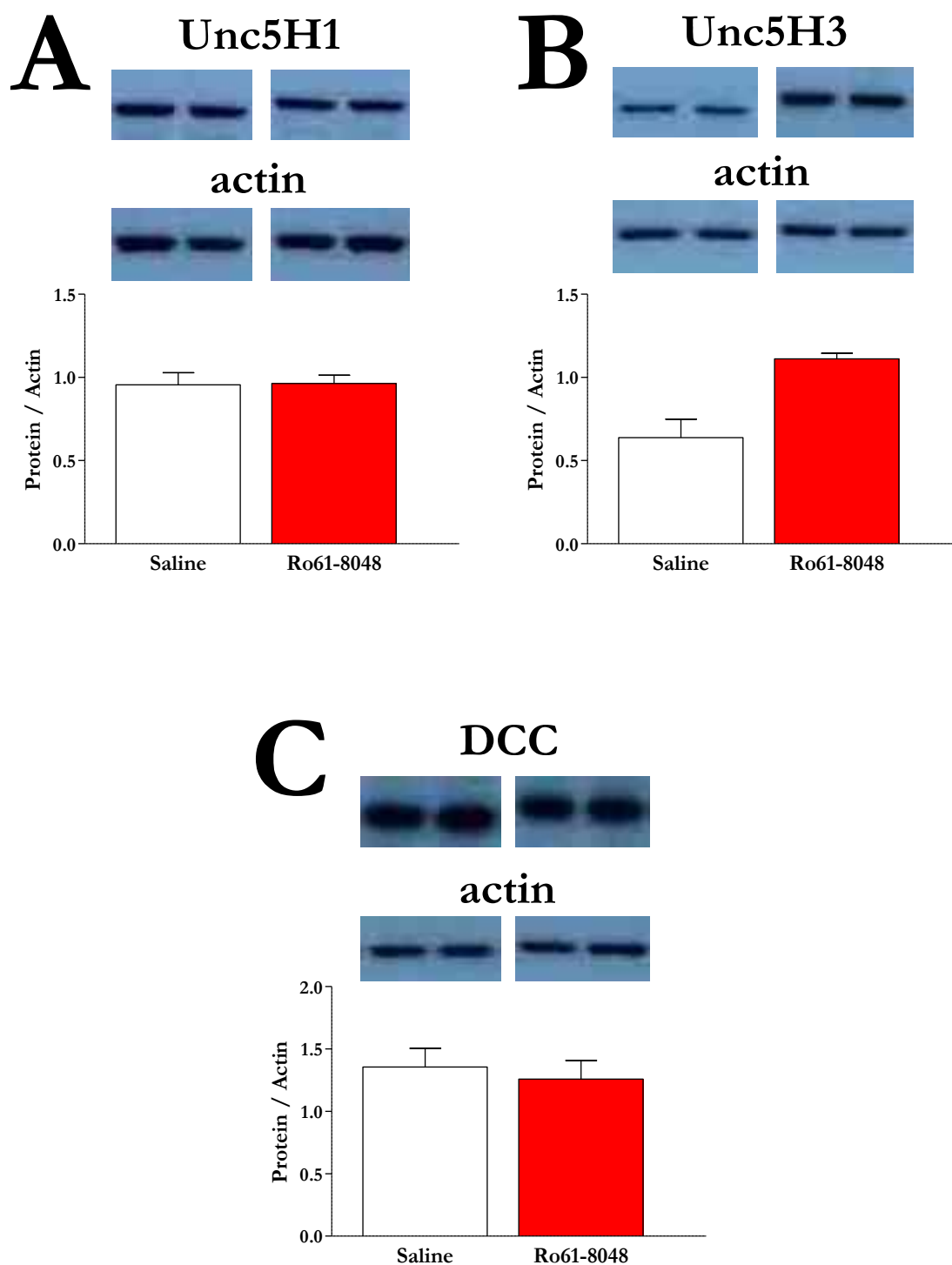
Interestingly, while DCX was significantly increased in the hippocampus, no change was seen in the cortex ( $p \geq 0.05$ ). Similarly, tyrosine hydroxylase (TH), 5HT-2c and  $\alpha$ -synuclein ( $\alpha$ -Syn) also remained unchanged ( $p \geq 0.05$ ), as seen in figure 3-30.

When the protein expression of VAMP-1 (synaptobrevin) was assessed in the cortex, its expression remained comparable with controls ( $p \geq 0.05$ ). Synaptic markers synaptotagmin (SynT) and synaptophysin (SynPhy) were also examined to find no significant difference existed between treatment groups ( $p \geq 0.05$ ) as seen in figure 3-31.



**Figure 3-28. GluN1, DISC-1, RhoA and RhoB expression in the P60 cortex.**

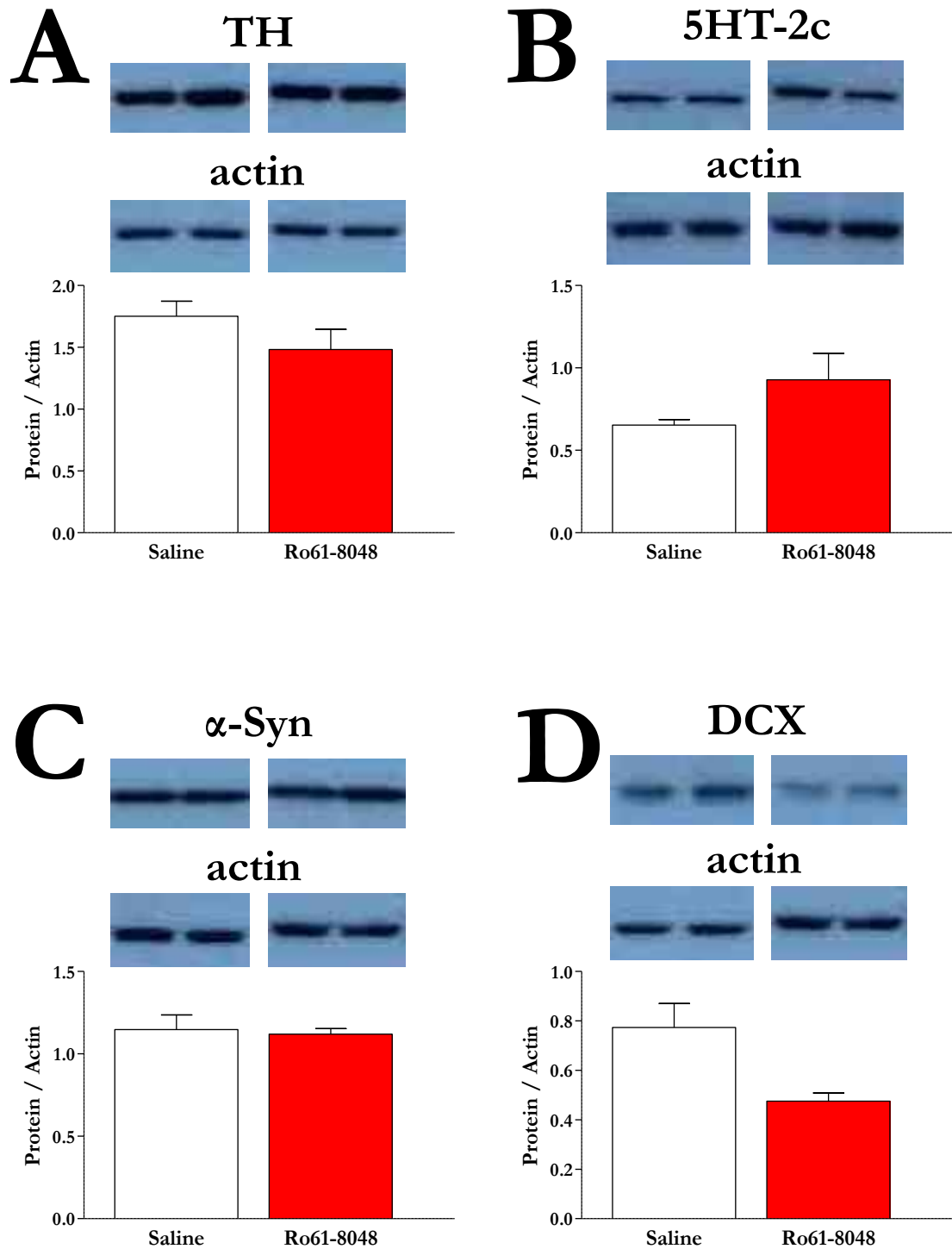
Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1, (C) RhoA and (D) RhoB in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the cortex by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ , except RhoA Ro61-8048 where  $n = 5$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-29. Unc5H1, Unc5H3 and DCC expression in the P60 cortex.**

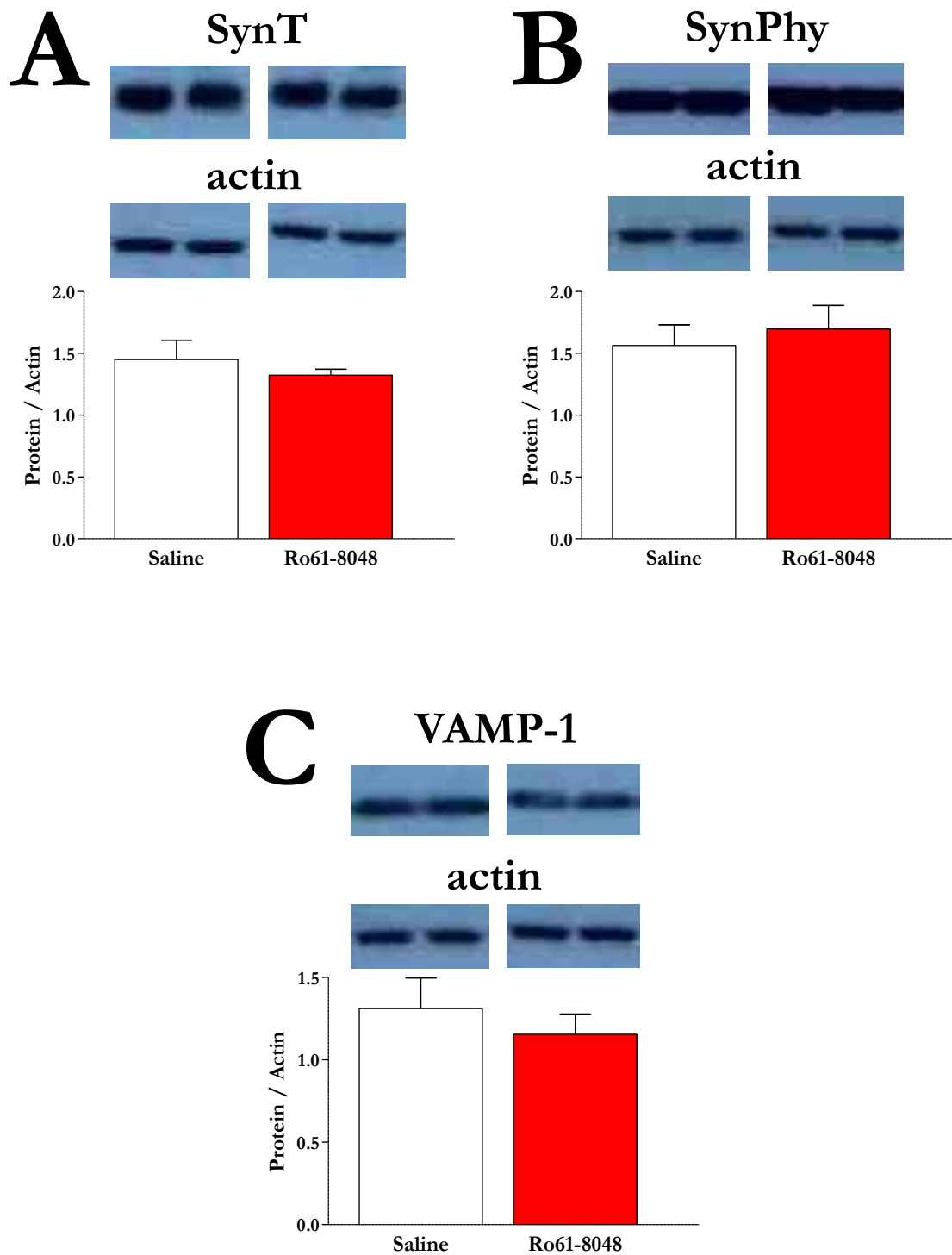
Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the cortex by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ , except Unc5H3 Ro61-8048 where  $n = 5$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.





**Figure 3-30. TH, 5HT-2c,  $\alpha$ -synuclein and DCX expression in the P60 cortex.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2c, (C) A-Syn and (D) DCX in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the cortex by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ , except TH Ro61-8048 where  $n = 5$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-31. Expression of synaptic proteins in the P60 cortex.**

Bar charts illustrate the quantified protein expression of (A) SynT, (B) SynPhy and (C) VAMP-1 in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the cortex by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.

### 3.6.3 Examination of Protein Markers in the P60 Midbrain

Midbrain pathology is seen in Parkinson's disease (PD) with a loss of catecholaminergic neurons in the substantia nigra including acetylcholine and dopamine, accompanied by deficits in the basal ganglia. Although Huntington's disease (HD) involves deficits in the hippocampus and cortex during later stages of the disease (Cowan and Raymond, 2006; Roze *et al.*, 2008), the predominant region affected includes lesions within the striatum (Heng *et al.*, 2009). Therefore, since the kynurenine pathway is intimately involved in the disease progression of these disorders, and may be involved in the initiation of Huntington's disease, the midbrain from P60 animals was dissected and processed for western blotting to examine changes in protein expression as an indication of developmental damage induced within this region following prenatal treatment with Ro61-8048. Although HD and PD remain neurodegenerative conditions as opposed to neurodevelopmental in origin, increasing evidence is beginning to associate the enhanced susceptibility to postnatal insults with the earlier onset of Parkinson's disease during adulthood when exposed prenatally to environmental factor such as infection or toxins (Fan *et al.*, 2011; Bobyn *et al.*, 2012). Furthermore, schizophrenia pathology involves an over-activity of the dopaminergic system within the substantia nigra, with neurochemical changes in the ventral tegmental area (VTA) and nucleus accumbens, making the midbrain a primary region for investigation. Therefore, since the kynurenine pathway is involved in a variety of disorders, the midbrain was examined for changes in protein expression

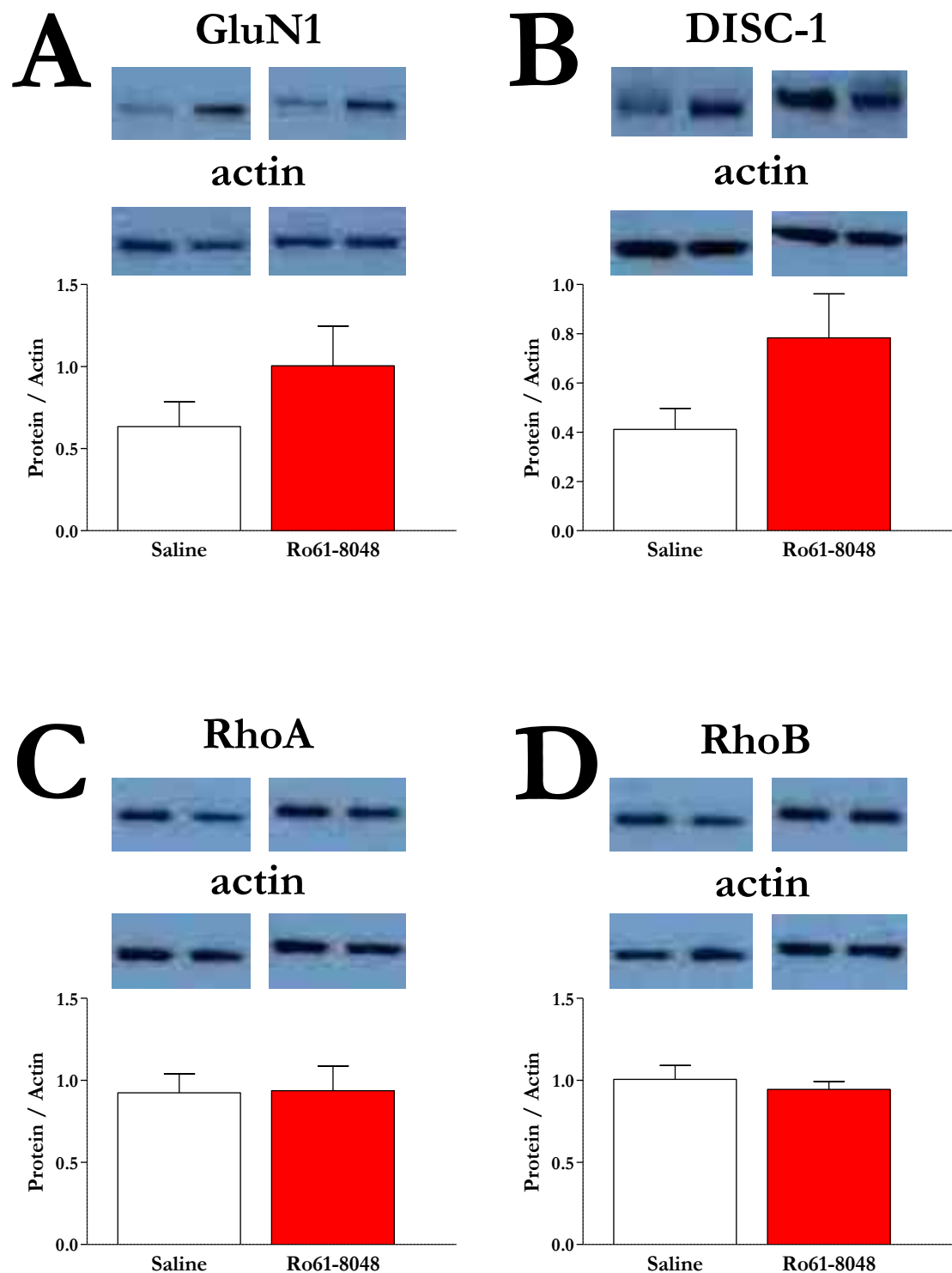
3.6.3.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats induces no changes in protein expression in the midbrain of postnatal day 60 animals

Examination of the midbrain by immunoblotting in P60 animals revealed no changes in the protein expression of GluN1, DISC-1, RhoA and RhoB ( $p > 0.05$ , figure 3-32).

Similarly, no significant change was seen in the protein expression of Unc5H1, Unc5H3 and DCC ( $p > 0.05$ , figure 3-33).

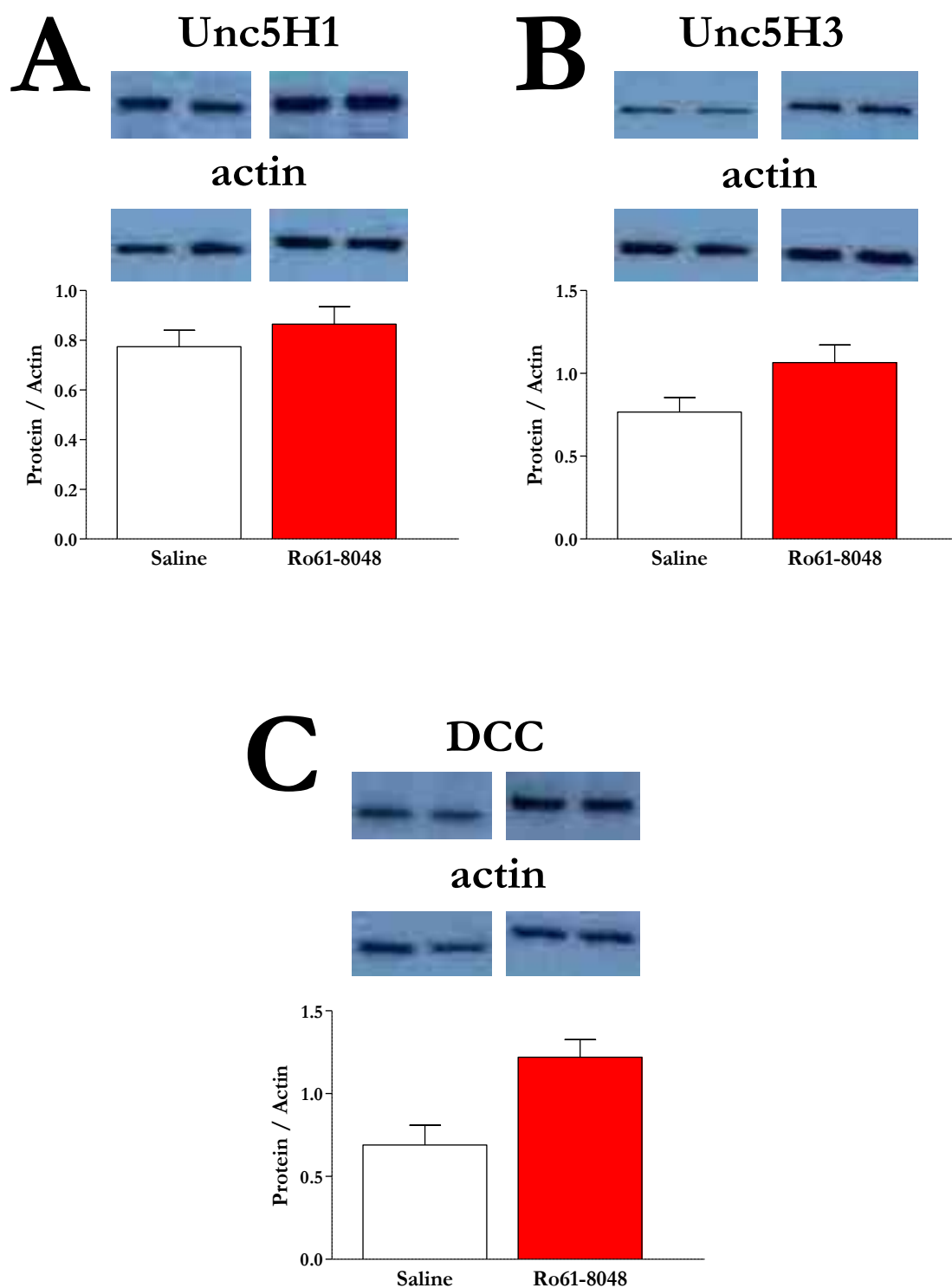
The protein expression of tyrosine hydroxylase (TH), 5HT-2c,  $\alpha$ -synuclein ( $\alpha$ -Syn) and doublecortin (DCX) also remained unchanged in the midbrain ( $p > 0.05$ ) as seen in figure 3-34.

When the protein expression of VAMP-1 (synaptobrevin) was assessed, its expression remained comparable with controls ( $p > 0.05$ ). Synaptic markers synaptotagmin (SynT) and synaptophysin (SynPhy) were also examined to find no significant difference existed between treatment groups ( $p > 0.05$ ) as seen in figure 3-35.



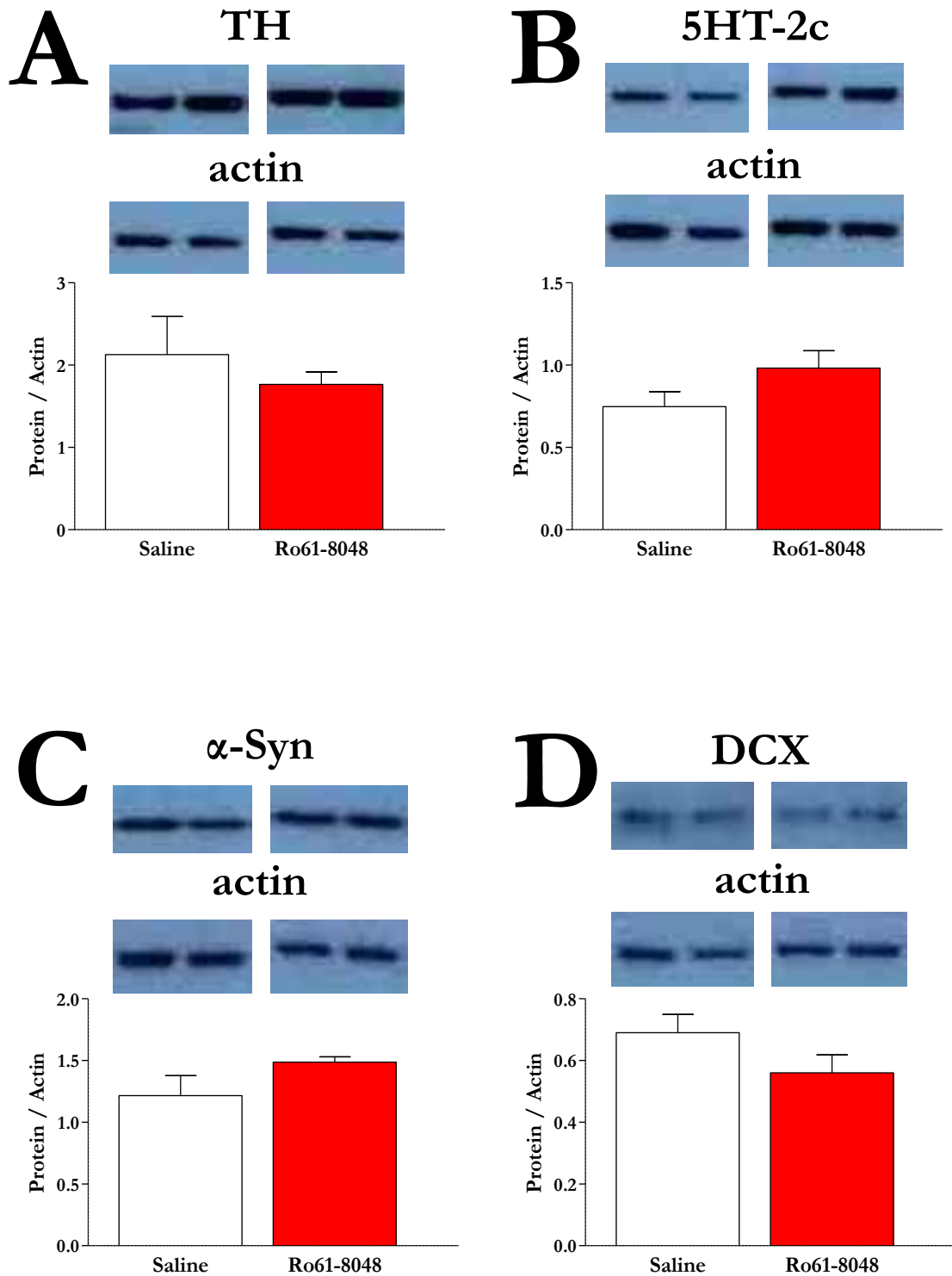
**Figure 3-32. GluN1, DISC-1, RhoA and RhoB expression in the P60 midbrain.**

Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1, (C) RhoA and (D) RhoB in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the midbrain by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ , except RhoA and RhoB saline where  $n = 5$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



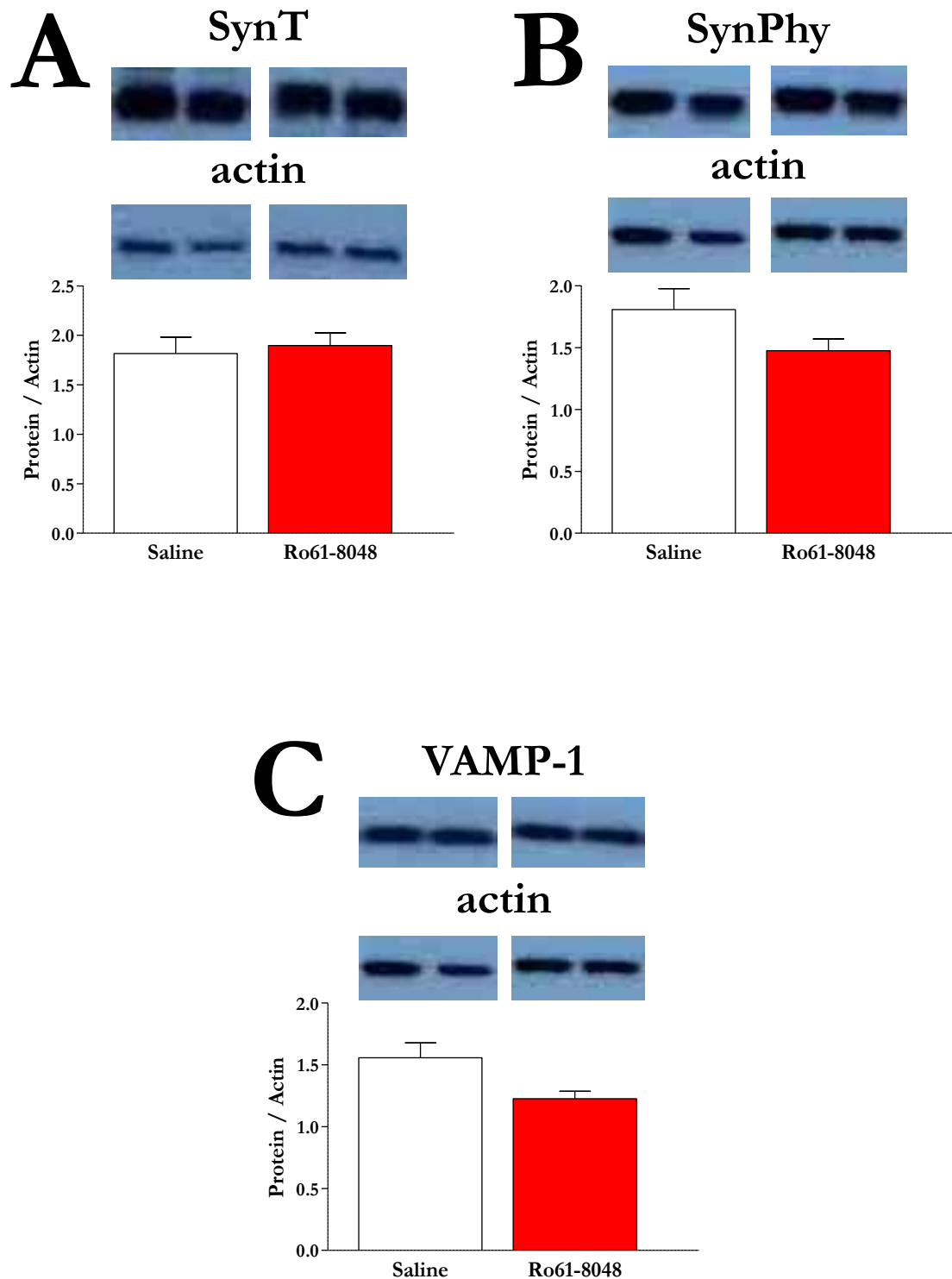
**Figure 3-33. Unc5H1, Unc5H3 and DCC expression in the P60 midbrain.**

Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the midbrain by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ , except Unc5H1 and Unc5H3 saline where  $n = 5$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-34. TH, 5HT-2c,  $\alpha$ -synuclein and DCX expression in the P60 midbrain.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2c, (C) A-Syn and (D) DCX in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the midbrain by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ , except for TH and 5HT-2C where  $n = 5-6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-35. Expression of synaptic proteins in the P60 midbrain.**

Bar charts illustrate the quantified protein expression of (A) SynT, (B) SynPhy and (C) VAMP-1 in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the midbrain by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 5$ , except for SynT where  $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



### 3.6.4 Examination of Protein Markers in the P60 Cerebellum

While the cerebellum has widely been associated in controlling motor co-ordination, it is increasingly becoming more apparent that the cerebellum may also mediate non-motor brain functions (Konarski *et al.*, 2005) involved in the pathophysiology of schizophrenia (Reviewed in Kyosseva, 2004; Andreasen and Pierson, 2008). This is supported by clinical evidence and structural neuroimaging studies associating cerebellar deficits to cognitive-related symptoms of schizophrenia (Lungu *et al.*, 2001). Predominant cerebellar hypoactivation has been reported, with the majority localised in the medial portion of the anterior lobe and lateral hemispheres of lobules IV-V of the cerebellum, and is proposed to contribute towards cognitive, emotional and executive functional processes in schizophrenia. The altered connectivity of neuronal connections identified in the cortex and hippocampus of schizophrenia patients have been extended to include the altered functional connectivity and organisation (Kannan *et al.*, 2009) of cortico-cerebellar connectivity, while the cerebellar-thalamic neuronal connections appear to be predominantly affected (Chen *et al.*, 2013). Neurochemically, transcription factors regulating genes in dopamine and glutamate pathways, including subunits of the NMDA receptor, are significantly altered in the cerebellum, inversely correlating with the negative symptoms in schizophrenia (Pinacho *et al.*, 2013). Disruptions in glutamate and GABA receptors and transmission have also been reported in the lateral cerebella of subjects with schizophrenia (Bullock *et al.*, 2008; Fatemi *et al.*, 2013), while these changes are attributed to contribute towards deficits in motor learning in patients with schizophrenia (Roder *et al.*, 2013). Interestingly, deficits in GABAergic signalling within the cerebellum may be partly responsible for disturbances in motor behaviour thought to result from abnormalities in the brain's timing mechanisms, due to reductions in GABAergic inhibitory interneuronal control of cellular activity (Teale *et al.*, 2013), and may contribute towards deficits in sensorimotor gating in schizophrenia. Since mounting evidence suggests cerebellar involvement in motor and non-motor brain functions during schizophrenia, we sought to examine protein changes within this region to see whether Ro61-8048 could adversely affect cerebellar function during adulthood.

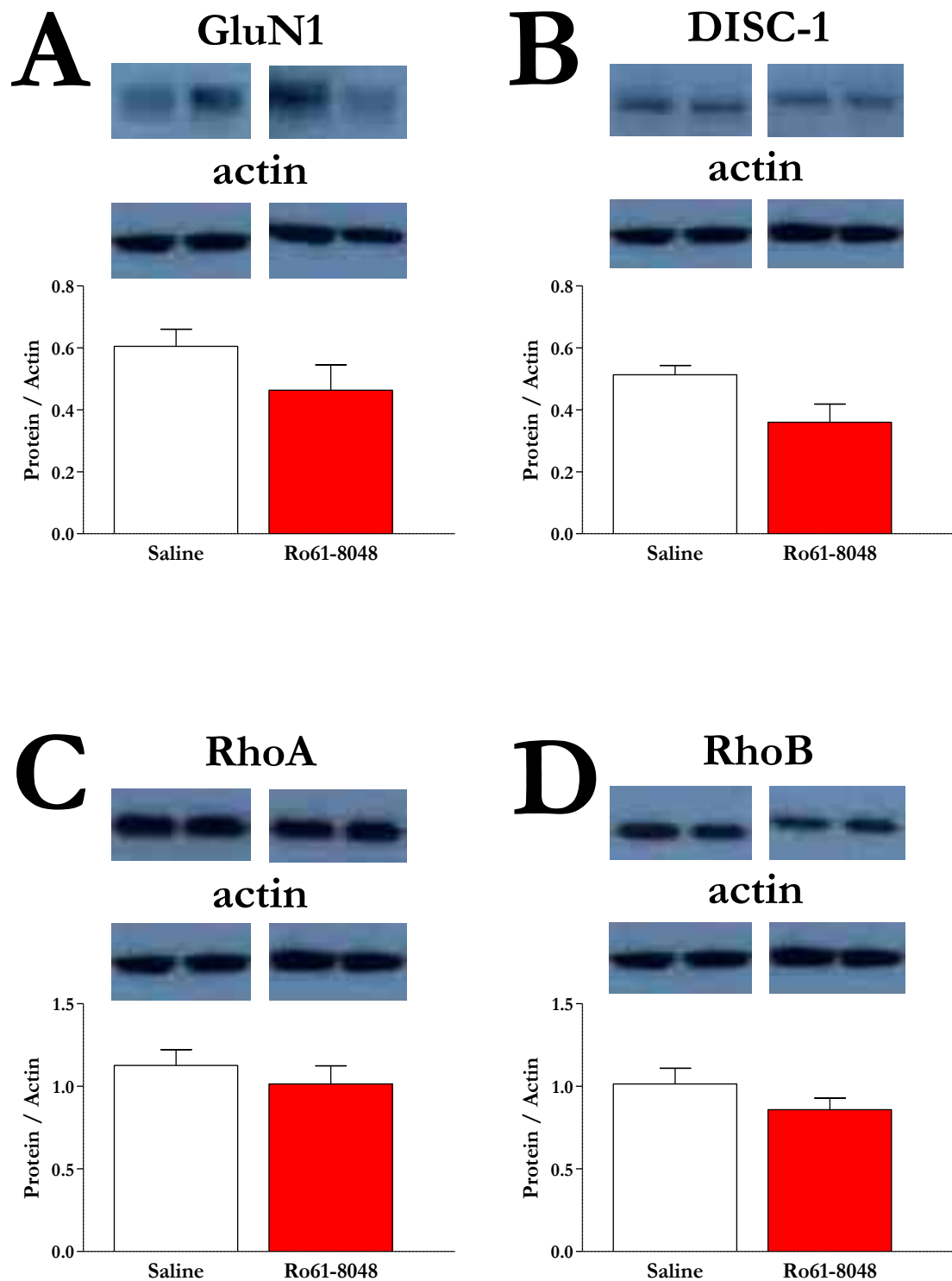
3.6.4.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats alters the expression of proteins related to axonal guidance in the cerebellum of postnatal day 60 animals

Examination of the cerebellum by immunoblotting revealed no changes in the protein expression of GluN1, DISC-1, RhoA and RhoB ( $p \geq 0.05$ ), as seen in figure 3-36.

While prenatal treatment to Ro61-8048 substantially reduced the protein expression of Unc5H3 ( $p < 0.01^{**}$ ) in the cerebellum, Unc5H1 and DCC expression remained comparable with controls ( $p \geq 0.05$ , figure 3-37).

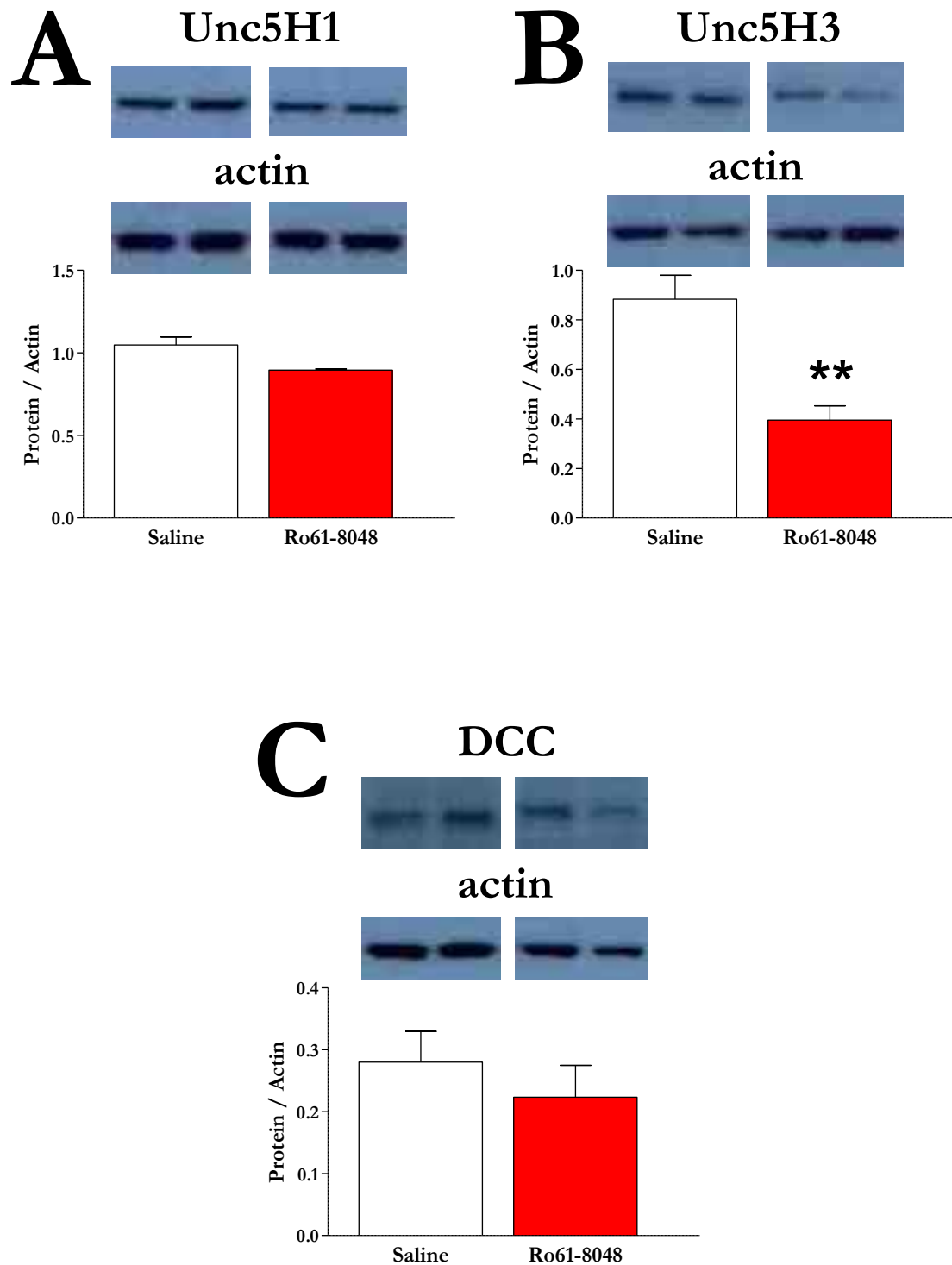
The protein expression of tyrosine hydroxylase (TH), 5HT-2c,  $\alpha$ -synuclein ( $\alpha$ -Syn) and doublecortin (DCX) remained unchanged in the cerebellum following prenatal treatment of Ro61-8048 ( $p \geq 0.05$ ), as seen in figure 3-38.

When the protein expression of VAMP-1 (synaptobrevin) was assessed in the cerebellum, its expression remained comparable with controls ( $p \geq 0.05$ ). The protein expression of synaptic markers synaptotagmin (SynT) and synaptophysin (SynPhy) were also examined to find no significant difference existed between treatment groups ( $p \geq 0.05$ ), as seen in figure 3-39.



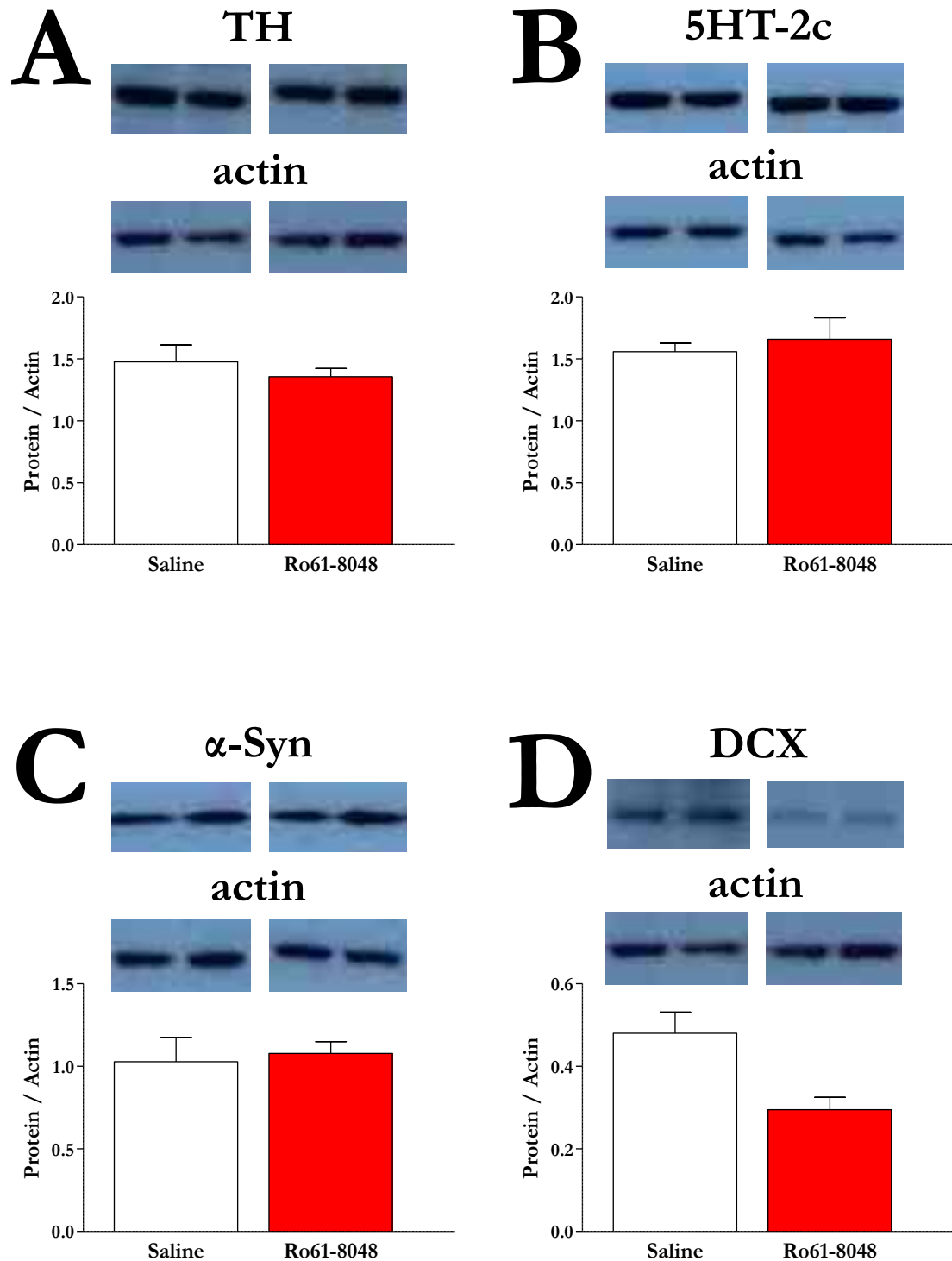
**Figure 3-36. GluN1, DISC-1, RhoA and RhoB expression in the P60 cerebellum.**

Bar charts illustrate the quantified protein expression of (A) GluN1, (B) DISC-1, (C) RhoA and (D) RhoB in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the cerebellum by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



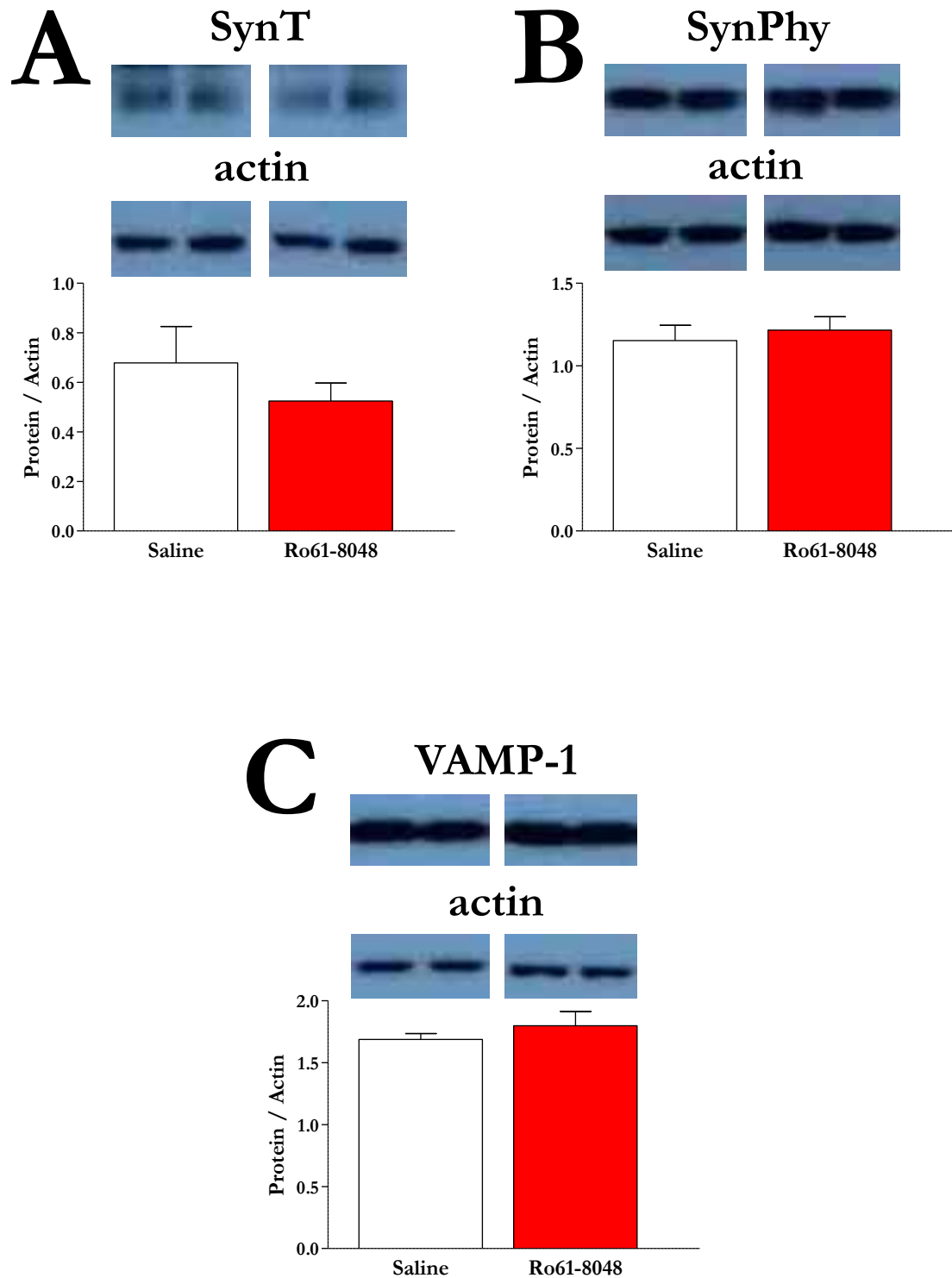
**Figure 3-37. Unc5H1, Unc5H3 and DCC expression in the P60 cerebellum.**

Bar charts illustrate the quantified protein expression of (A) Unc5H1, (B) Unc5H3 and (C) DCC in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the cerebellum by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-38. TH, 5HT-2c,  $\alpha$ -synuclein and DCX expression in the P60 cerebellum.**

Bar charts illustrate the quantified protein expression of (A) TH, (B) 5HT-2c, (C) A-Syn and (D) DCX in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the cerebellum by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.



**Figure 3-39. Expression of synaptic proteins in the P60 cerebellum.**

Bar charts illustrate the quantified protein expression of (A) SynT, (B) SynPhy and (C) VAMP-1 in the brains of rat offspring following maternal exposure to 0.9 % saline or 100 mg/kg Ro61-8048 on gestation days E14, E16 and E18. Animals were sacrificed at P60 and their brains removed and dissected to examine any relative or absolute changes in protein expression analysed in the cerebellum by immunoblotting. Results are expressed as  $\pm$  s.e.mean ( $n = 6$ ) displaying arbitrary units of optical density expressed as the ratio of target protein to actin. Statistical comparisons were made using a one way ANOVA with Bonferoni correction for multiple comparisons between groups of animals born to mothers treated with saline and groups of animals born to mothers treated with Ro61-8048. Representative examples of typical protein bands obtained from the western blots along with their corresponding actin, which was used as a loading control, are presented above each bar chart.

A summary of all changes in protein expression in embryo brains, P21 and P60 animals following prenatal treatment with Ro61-8048 can be seen in table 3-2 and table 3-3.

**Table 3-2. Summary of protein changes following Ro61-8048 treatment until P21**

Protein Marker	Embryo E18 5h	Embryo E18 24h	Postnatal Day P21
GluN1	x	x	x
DISC-1	x	x	x
PSD-95	-	-	x
RhoA	x	x	x
RhoB	x	x	p=<0.05* (dec. in Ro61-8048)
Unc5H1	x	x	x
Un5H3	x	x	x
DCC	x	x	x
TH	x	x	x
5HT-2 <sub>c</sub>	x	x	x
A-Syn	x	x	x
DCX	x	x	x
VAMP-1	x	x	x
SynT	-	-	x
SynPhy	-	-	x

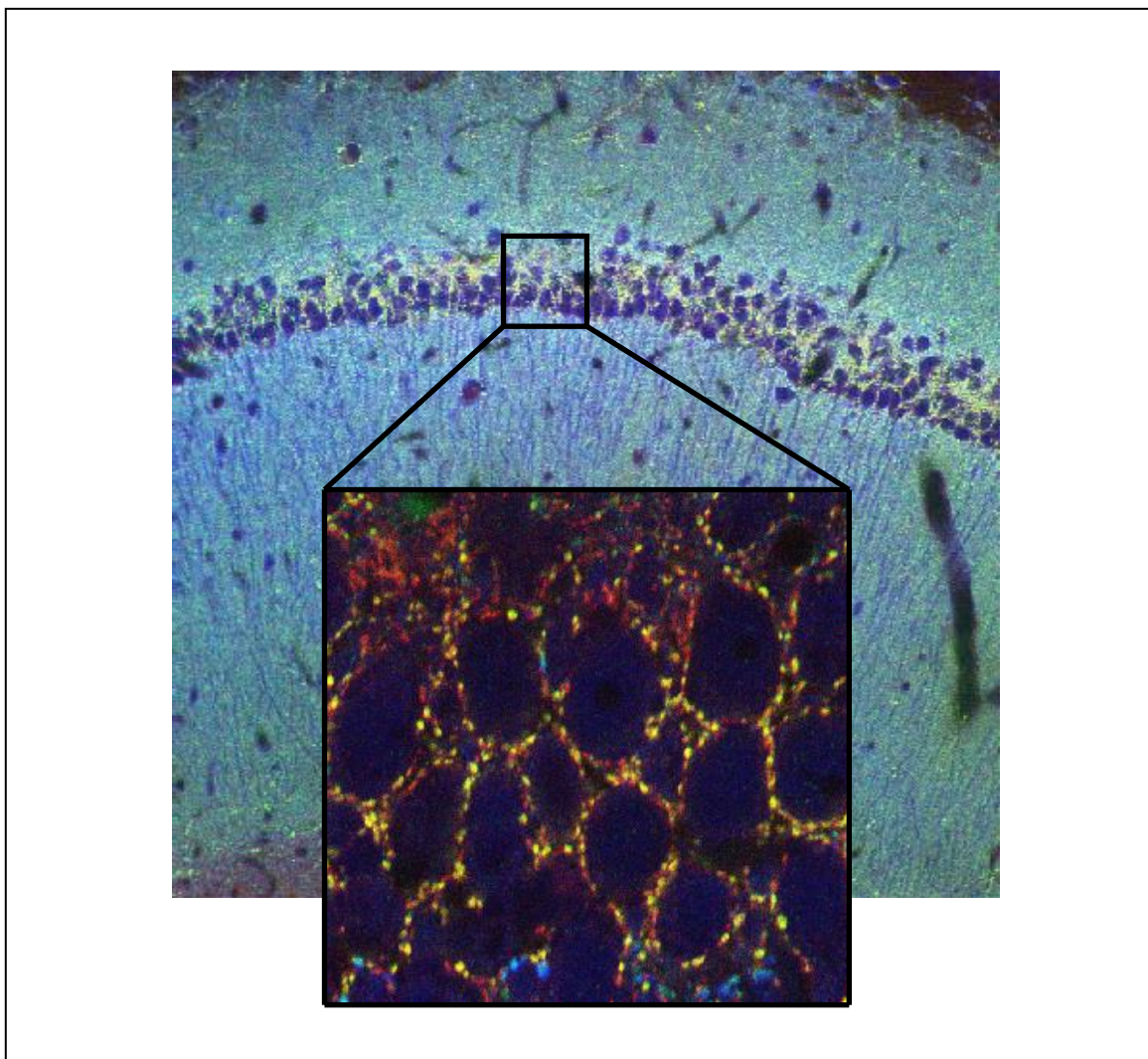
**Table 3-3. Summary of protein changes following Ro61-8048 treatment at P60**

Protein Marker	Hippocampus	Cortex	Midbrain	Cerebellum
GluN1	x	x	x	x
DISC-1	x	P=<0.05* (inc. in Ro61-8048)	x	x
RhoA	x	x	x	x
RhoB	x	x	x	x
Unc5H1	x	x	x	x
Un5H3	x	x	x	p=<0.01** (dec. in Ro61-8048)
DCC	x	x	x	x
TH	x	x	x	x
5HT-2 <sub>c</sub>	x	x	x	x
A-Syn	x	x	x	x
DCX	P=<0.05* (inc. in Ro61-8048)	x	x	x
VAMP-1	x	x	x	x
SynT	x	x	x	x
SynPhy	x	x	x	x

### 3.7 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats affects glutamatergic transporters and spine density in the CA1 hippocampus during adulthood

Several groups have provided evidence of altered excitatory and inhibitory signalling during schizophrenia occurring within the hippocampus during adulthood, with reports of neurochemical changes in gene and protein expression. The initial hypothesis remained that altered activity along the kynurenine pathway prenatally would induce significant alterations in the concentrations of kynurenic acid thereby affecting NMDA receptor activation during development. Since this mechanism proposed a loss of NMDA function during development, a phenomenon associated with abnormalities in behaviour and changes in excitatory and inhibitory neuronal function (Harris *et al.*, 2003; Uehara *et al.*, 2009), the relative densities of excitatory (VGLUT-1, VGLUT-2) and inhibitory (VGAT) transporters were examined as well as their co-localisation with the synaptic marker vesicle associated membrane protein (VAMP)-1, to identify whether prenatal interference with kynurenines could modify neuronal function during adulthood. Furthermore, since the hippocampus is a principal region affected during schizophrenia (Harrison *et al.*, 2006; Shan *et al.*, 2013), the relative densities were examined in the stratum pyramidale layer of the CA1 region. This location can be seen in figure 3-40. Images were acquired using a x60 objective lens with oil immersion and a zoom factor of 3. Parameters controlling for the contrast and laser power of each channel were adjusted within the maximum range to prevent fusion of puncta. Technical replicates were taken to control for the variation in counting the number of transporters per section, and a total of 3 technical replicates (sections) were averaged per biological replicate (animal). A *t* test was then performed on the average of 8 biological replicates (animals) to observe whether differences in the number of transporters represented a true change between treatment groups.



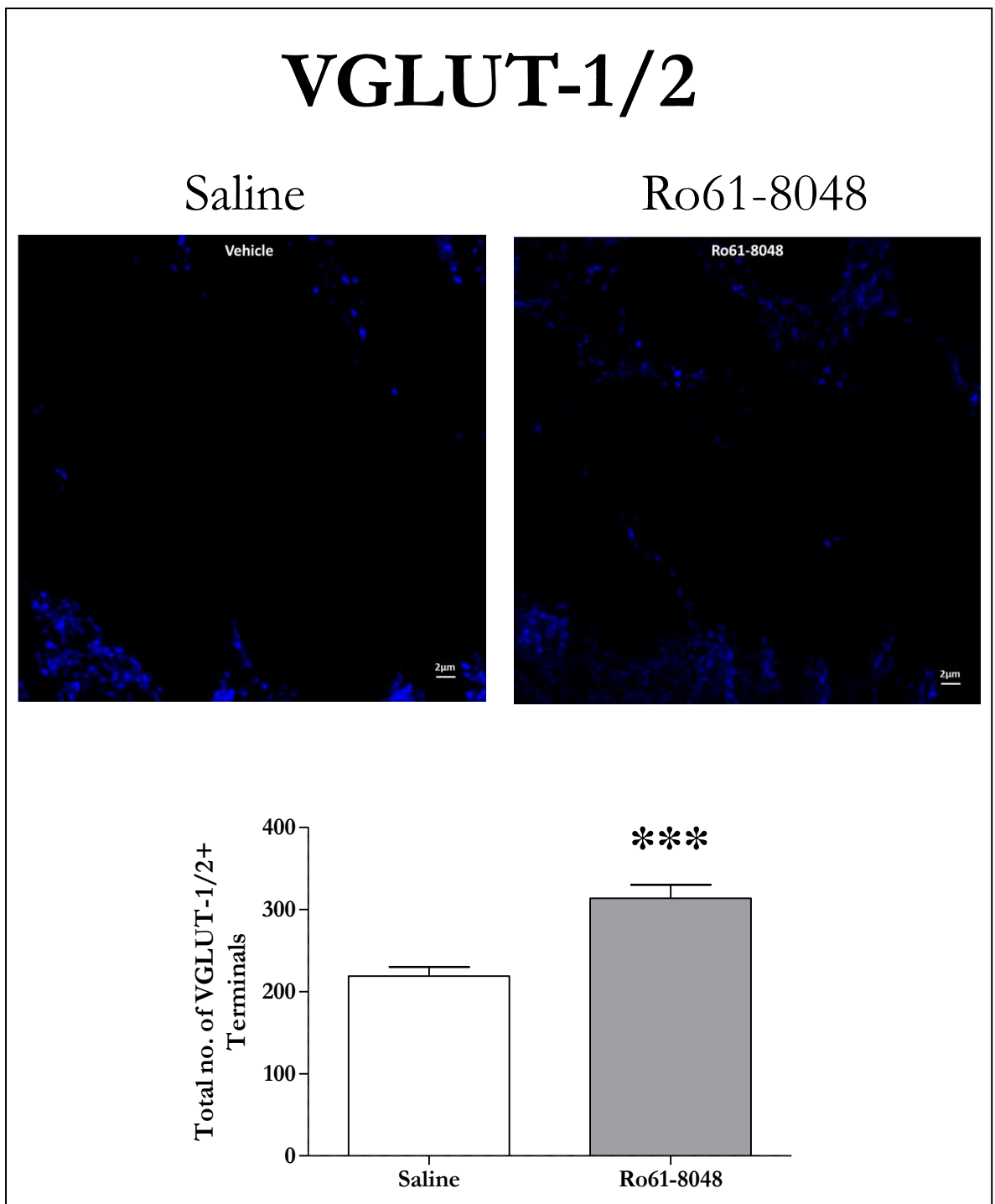


**Figure 3-40. The CA1 region of the hippocampus analysed for punctate staining.**

Punctate staining for VGLUT-1, VGLUT-2, VGAT and VAMP-1 were counted manually within the CA1 region of the hippocampus as an indication of glutaminergic signalling within the hippocampus. Images were acquired using a x60 objective lens with oil immersion and a zoom factor of 3.

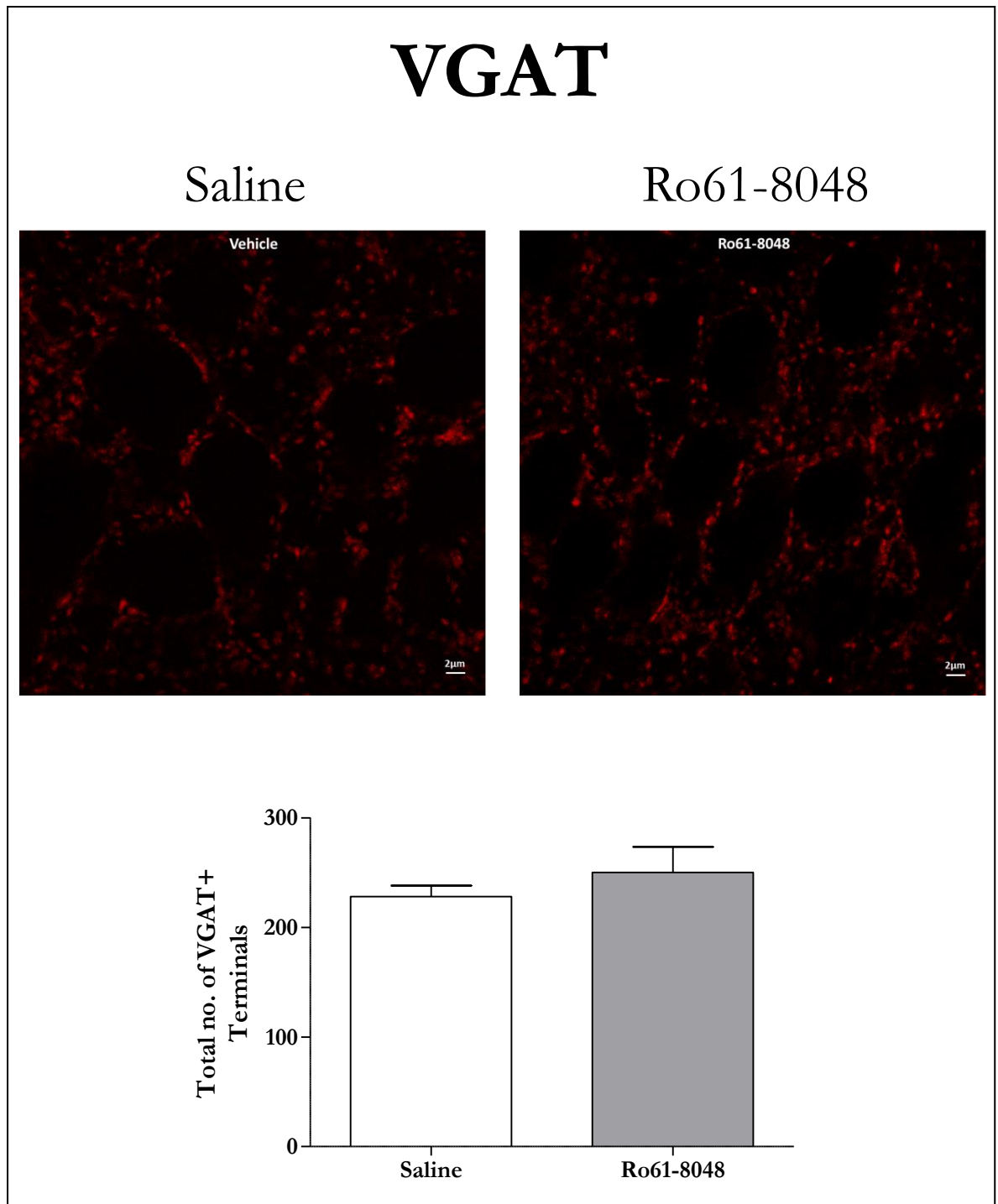
*3.7.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats increases the density of excitatory transporters in nerve terminals of the stratum pyramidale layer of the hippocampus in postnatal day 60 animals*

Confocal analysis of hippocampal sections in the CA1 region revealed a highly significant increase in the densities of excitatory VGLUT-1 and VGLUT-2 transporters ( $P=0.0003^{***}$ ; figure 3-41) in the stratum pyramidale of P60 animals born to mothers treated prenatally with Ro61-8048, while the relative density of inhibitory VGAT transporters remained unchanged ( $p=0.41$ , figure 3-42). To ascertain that changes in transporters were not due to changes in synaptic connections, the relative density of VAMP-1 was also examined to reveal no change between the treatment group and control animals ( $p=0.58$ , figure 3-43). Finally, no changes were seen in co-localisation between VAMP-1 and VGLUT-1 and VGLUT-2 ( $p=0.22$ , figure 3-44) and co-localisation between VAMP-1 and VGAT ( $p=0.82$ , figure 3-45).



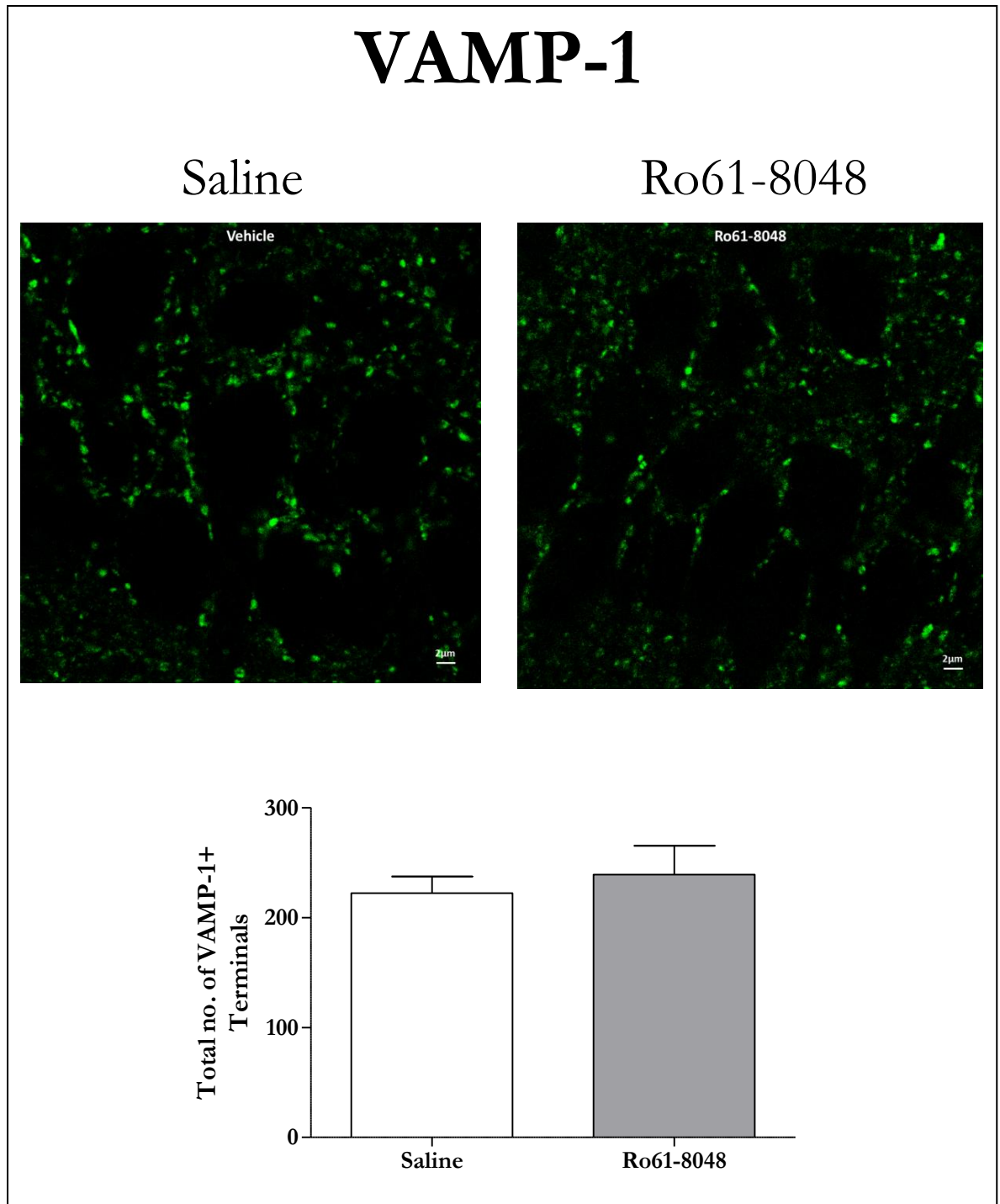
**Figure 3-41. VGLUT-1/2 immunoreactivity in the CA1 region of the hippocampus.**

A representative image of the staining obtained from vesicular glutamate transporters-1 and 2 (VGLUT-1/2) in the stratum pyramidale layer of the hippocampus. Punctate staining was counted manually within a box size of 50µm x 50µm. 3 sections per animal from a total of 8 animals contributed towards the statistical analysis (n = 8 animals) where results are expressed as +/- s.e.mean. The data was analysed using an unpaired *t* test and a probability value of 0.05 was adopted as the working criterion for significance. Scale bars are set to 2µm.



**Figure 3-42. VGAT immunoreactivity in the CA1 region of the hippocampus.**

A representative image of the staining obtained from vesicular GABA transporters (VGAT) in the stratum pyramidale layer of the hippocampus. Punctate staining was counted manually within a box size of  $50\mu\text{m} \times 50\mu\text{m}$ . 3 sections per animal from a total of 8 animals contributed towards the statistical analysis ( $n = 8$  animals) where results are expressed as  $\pm$  s.e.mean. The data was analysed using an unpaired  $t$  test and a probability value of 0.05 was adopted as the working criterion for significance. Scale bars are set to  $2\mu\text{m}$ .



**Figure 3-43. VAMP-1 immunoreactivity in the CA1 region of the hippocampus.**

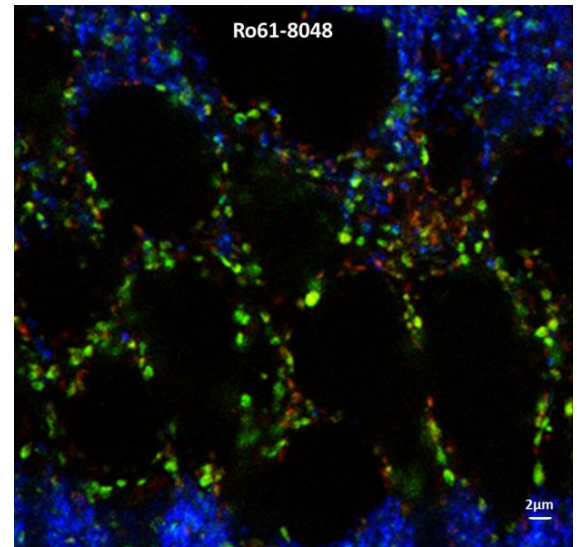
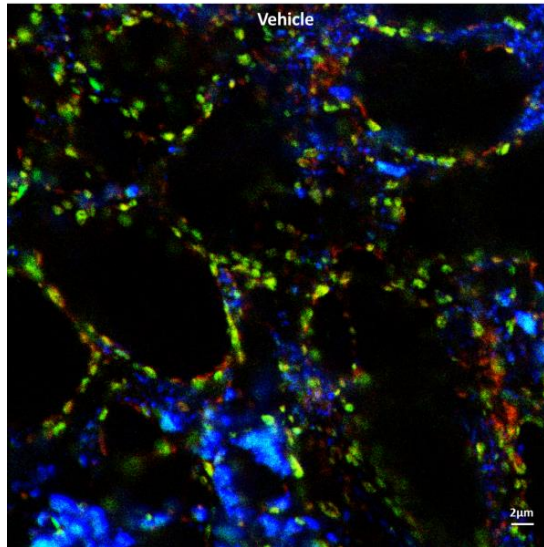
A representative image of the staining obtained from vesicle-associated membrane protein-1 (VAMP-1) protein in the stratum pyramidale layer of the hippocampus. Punctate staining was counted manually within a box size of 50µm x 50µm. 3 sections per animal from a total of 8 animals contributed towards the statistical analysis (n = 8 animals) where results are expressed as +/- s.e.mean. The data was analysed using an unpaired *t* test and a probability value of 0.05 was adopted as the working criterion for significance. Scale bars are set to 2µm.



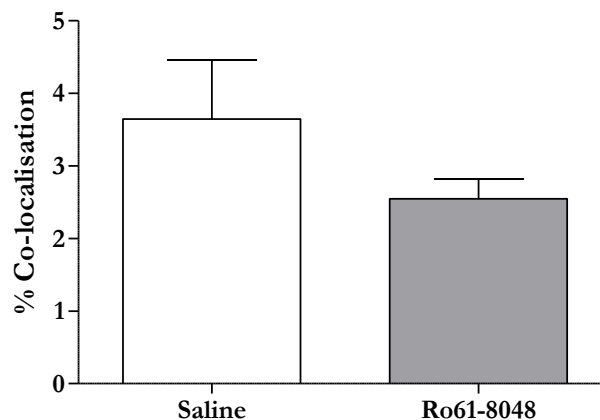
# [VAMP-1 :: VGLUT-1/2]

Saline

Ro61-8048



VAMP-1  
Co-localised with  
VGLUT-1/2



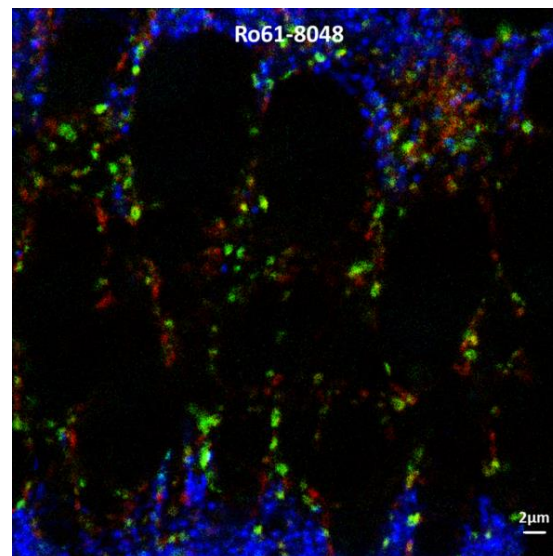
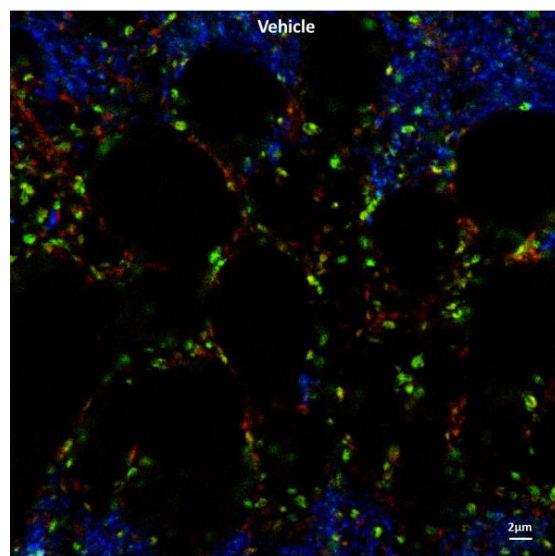
**Figure 3-44. VAMP-1 co-localisation with VGLUT-1/2 in the CA1 region of the hippocampus.**

A representative image of the staining obtained from vesicle-associated membrane protein-1 (VAMP-1) co-localised with vesicular glutamate transporters 1 and 2 (VGLUT-1/2) in the stratum pyramidale layer of the hippocampus. Images for VAMP-1 and VGLUT-1/2 were merged to assess their co-localisation and punctate staining was counted manually within a box size of 50µm x 50µm. Punctate staining was considered double-labelled when the overlap was complete or occupied most of the puncta while maintaining morphological similarity. 3 sections per animal from a total of 8 animals contributed towards the statistical analysis (n = 8 animals) where results are expressed as +/- s.e.mean. The data was analysed using an unpaired *t* test and a probability value of 0.05 was adopted as the working criterion for significance. Scale bars are set to 2µm.

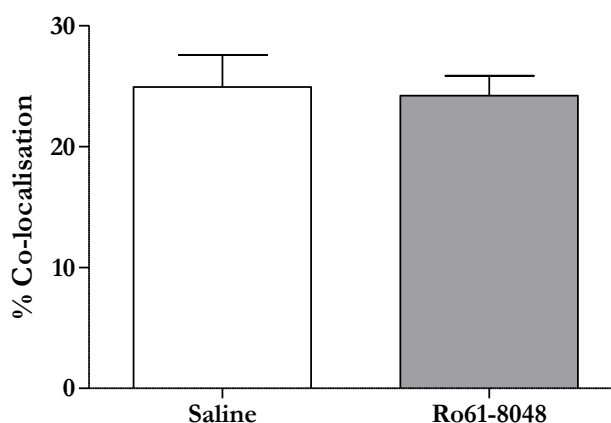
# [VAMP-1 :: VGAT]

Saline

Ro61-8048



VAMP-1  
Co-localised with  
VGAT



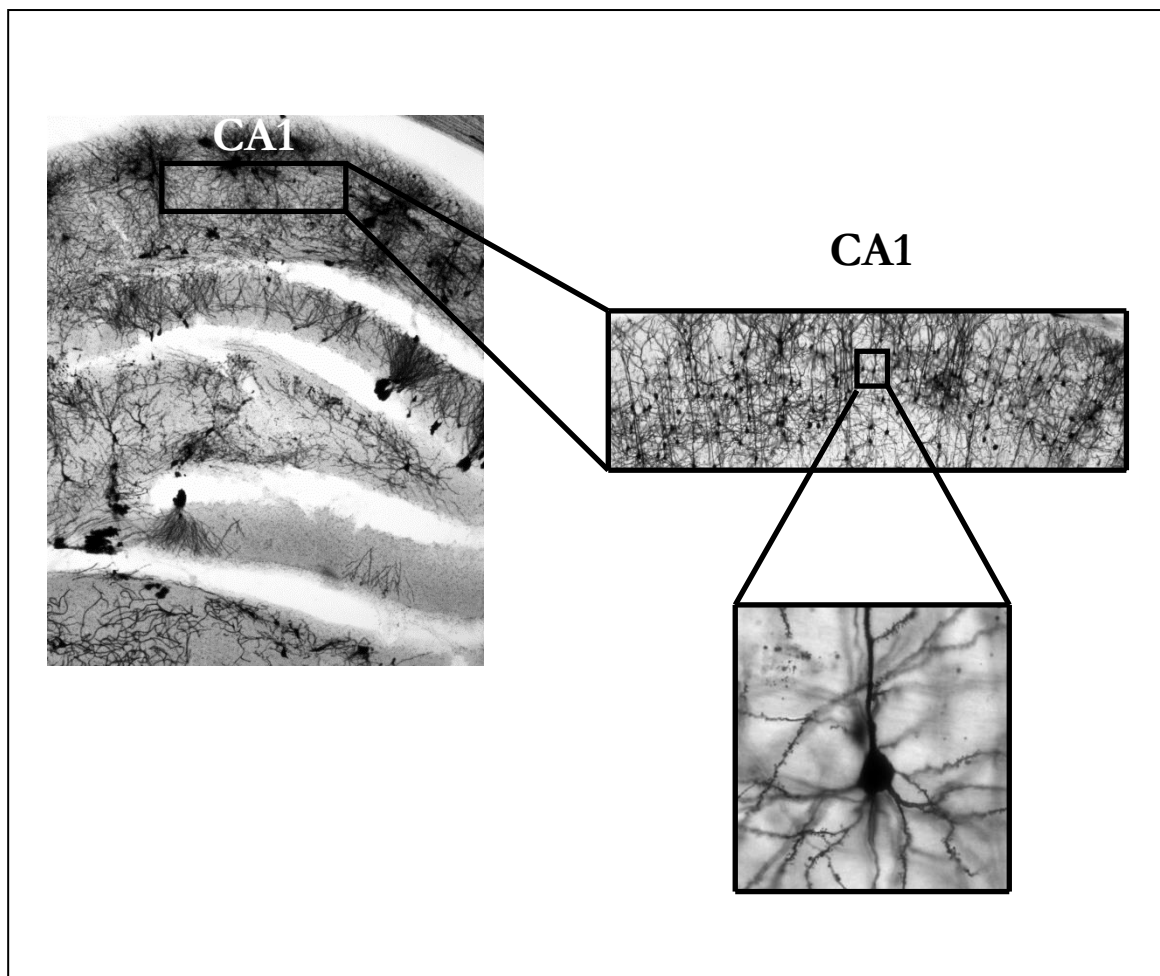
**Figure 3-45. VAMP-1 co-localisation with VGAT in the CA1 region of the hippocampus.**

A representative image of the staining obtained from vesicle-associated membrane protein-1 (VAMP-1) co-localised with vesicular GABA transporters (VGAT) in the stratum pyramidale layer of the hippocampus. Images for VAMP-1 and VGAT were merged to assess their co-localisation and punctate staining was counted manually within a box size of 50µm x 50µm. Punctate staining was considered double-labelled when the overlap was complete or occupied most of the puncta while maintaining morphological similarity. 3 sections per animal from a total of 8 animals contributed towards the statistical analysis (n = 8 animals) where results are expressed as +/- s.e.mean. The data was analysed using an unpaired *t* test and a probability value of 0.05 was adopted as the working criterion for significance. Scale bars are set to 2µm.

### *3.7.2 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats affects hippocampal spine density*

Many disorders involving a neurodevelopmental aetiology appear to be associated with significant impairments in neuronal morphology including alterations in dendritic spines. Since many changes in protein expression in postnatal animals, induced by Ro61-8048, have known roles in neuronal maturation and development, while some are directly related to regulate spine density, we sought to examine whether these changes in protein expression were also accompanied by alterations in dendritic spines. Furthermore, since dendritic spines represent the majority of excitatory inputs into a cell, and the examination of VGLUT-1 and VGLUT-2 staining in the dendritic layer of the CA1 region of the hippocampus was not feasible due to the extremely high density of punctate staining observed, the relative densities of dendritic spines were assessed as an indication of the level of excitatory input received in these cells. The CA1 region of the hippocampus analysed for spine density measurements can be seen in figure 3-46. Therefore, pregnant rats were administered an intraperitoneal injection of 100 mg/kg Ro61-8048 during E14, E16 and E18 and allowed to litter normally. Animals were taken from the home cage at P60, euthanized and had their brains extracted for processing by golgi analysis.





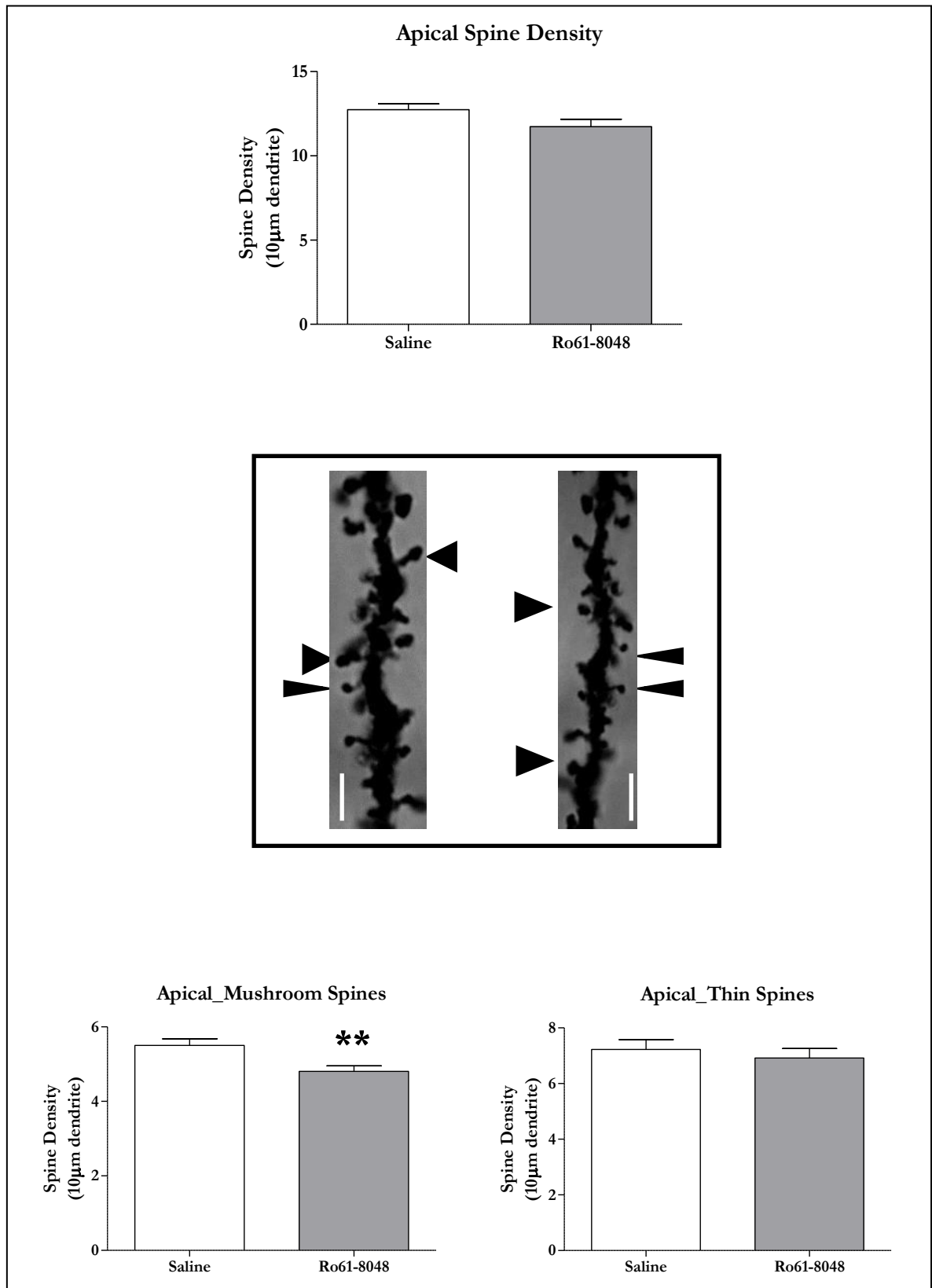
**Figure 3-46. CA1 region of the hippocampus for golgi analysis of dendritic spines.**

Since dendritic spines represent the majority of excitatory inputs into a cell, their relative densities were assessed, using the golgi impregnation method, as an indication of the level of excitatory input received in these cells.

3.7.2.1 Prenatal inhibition of KMO with Ro61-8048 in pregnant rats  
reduces spine density of pyramidal cells in the CA1 region of the  
hippocampus in postnatal day 60 animals

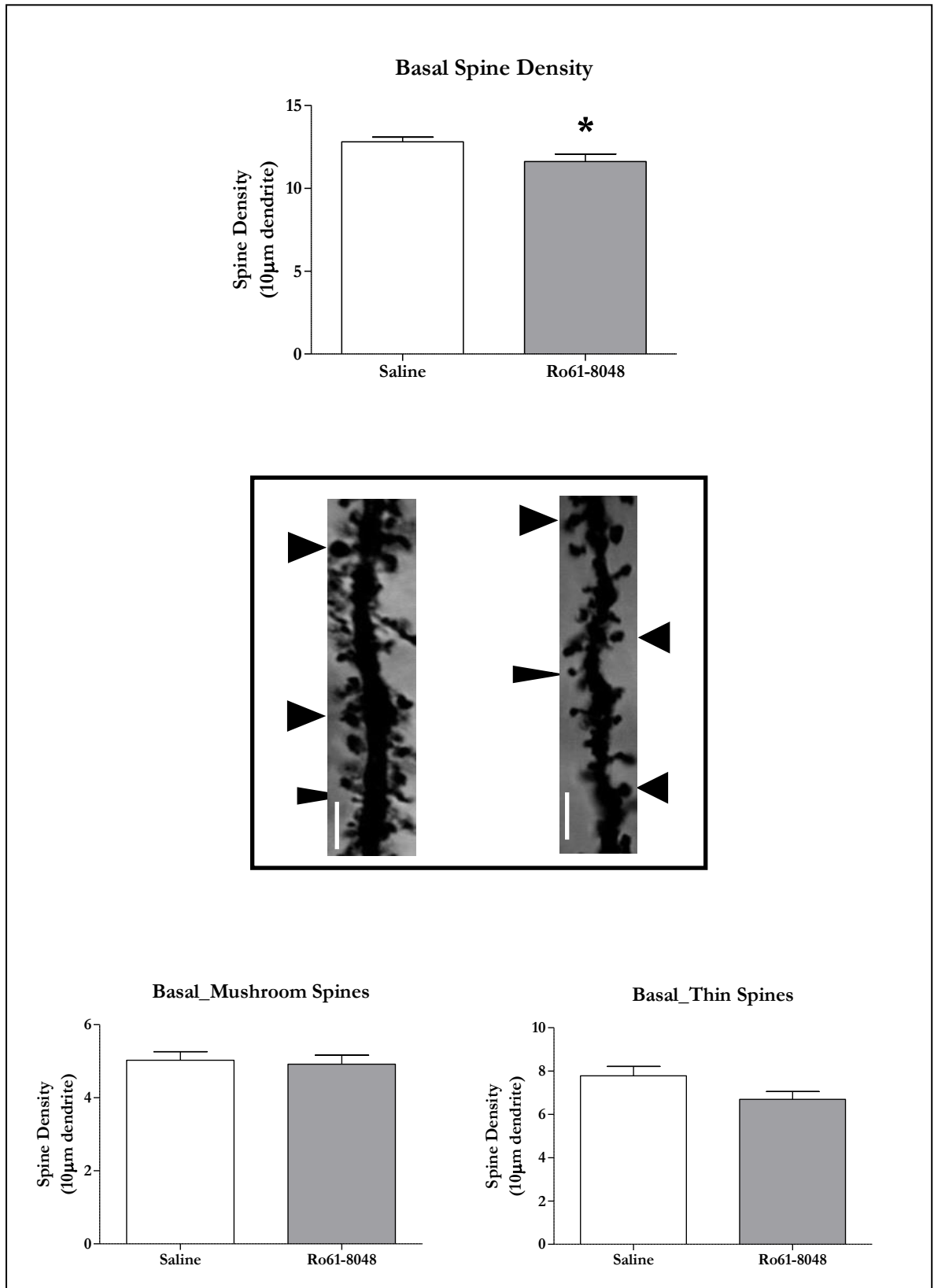
Analysis of spine density on pyramidal cells in the CA1 region of the hippocampus revealed no change in apical dendritic spines ( $p=0.08$ ), however, when thick mushroom and thin spines were analysed separately, a significant reduction was seen in mushroom spines ( $p=0.007^{**}$ ) while the relative density of thin spines remained comparable with controls ( $p=0.54$ ), as seen in figure 3-47.

Analysis of basal dendrites on pyramidal cells displayed significantly reduced densities of dendritic spines ( $p=0.035^{*}$ ) in groups of animals born to mothers treated prenatally with Ro61-8048. However, when mushroom and thin spines were analysed alone, no significant difference existed between the two subtypes ( $p=0.75$  and  $p=0.07$  respectively), as seen in figure 3-48.



**Figure 3-47. Relative spine density of apical dendrites in the CA1 hippocampus.**

Dendritic spines were manually counted under a light microscope using a x100 objective lens. Spine densities of mushroom spines (thick arrows) in apical dendrites were significantly reduced in drug-treated animals while thin spines (thin arrows) remained unchanged. Spines were counted per 10µm length of dendrite, while 3 dendritic segments per cell and a total of 4 cells per animal from 9 animals per treatment group ( $n = 9$  animals) contributed towards the analysis. The results are expressed as  $\pm$  s.e.mean. The data was analysed using an unpaired  $t$  test and a probability value of 0.05 was adopted as the working criterion for significance. Scale bar is set to 2µm.



**Figure 3-48. Relative spine density of basal dendrites in the CA1 hippocampus.**

Dendritic spines were manually counted under a light microscope using a x100 objective lens. Overall spine densities of basal dendrites were significantly reduced in drug-treated animals, while the selective density of mushroom (thick arrows) and thin spines (thin arrows) remained comparable with controls. Spines were counted per 10µm length of dendrite, while 3 dendritic segments per cell and a total of 4 cells per animal from 9 animals per treatment group ( $n = 9$  animals) contributed towards the analysis. The results are expressed as  $\pm$  s.e.mean. The data was analysed using an unpaired  $t$  test and a probability value of 0.05 was adopted as the working criterion for significance. Scale bar is set to 2µm.

## 1.0 Overview

Correct neuronal development in the developing foetus is paramount to the health and well-being of the offspring, critically dependent upon accurate neuronal differentiation, migration and precise axonal guidance leading to the formation of functional synapses on dendritic spines. However, maternal pregnancy (gestation) is a very vulnerable period for the developing embryo, and several risk factors have been identified capable of interrupting these physiological processes and damaging foetal development resulting in changes in brain neurochemistry, neuronal morphology and behaviours during adulthood (Schlotz and Phillips, 2009). While the effects of maternal smoking (Lobel *et al.*, 2008) alcohol (Sayal *et al.*, 2007; Haley *et al.*, 2006) and drug exposure (Bennet *et al.*, 2008; Goldschmidt *et al.*, 2008) as risk factors for neurodevelopmental disorders are widely appreciated, risk factors including maternal malnutrition, stress and infection (Mednick *et al.*, 1988; Adams *et al.*, 1993; Brown *et al.*, 2009; Eriksen *et al.*, 2009) are increasingly become more prevalent. Indeed, the influenza epidemic saw the subsequent rise in schizophrenia which has been associated with pregnant mothers contracting the virus, while the epidemic of encephalitis lethargica may have been a contributing factor for the increased incidence of Parkinson's disease. The effects of maternal immune activation as a risk factor for schizophrenia is now accepted suggesting an increasingly popular theory of a neurodevelopmental aetiology for psychiatric disorders like schizophrenia. While many studies have clearly demonstrated in rodent models, cognitive and behavioural abnormalities emerging from immune-challenged mothers (Zuckerman and Weiner, 2005; Romero *et al.*, 2007; Meyer *et al.*, 2007; Meyer *et al.*, 2008a; Lipina *et al.*, 2013), often subtle but reminiscent of those observed in schizophrenia (Fatemi and Folsom, 2009), very little is known regarding the mechanisms involved and the neurochemical alterations underlying these deficits. Although cytokines are noted for mediating the link between maternal immune activation and the subsequent risk in schizophrenia (Smith *et al.*, 2007; Cui *et al.*, 2009), it fails to explain how a relatively transient inflammatory reaction during pregnancy, rarely persisting beyond 24 h (Cunningham *et al.*, 2007), is capable of precipitating long-term changes in brain and behaviour. Alongside cytokines producing a short-term direct action on the developing embryo, it is most likely that long-term alterations are due to the subsequent sequence of events involving alterations in intracellular signalling cascades, receptor and transmitter systems are initiated by the initial insult. To identify some of these neurochemical changes, this study sought to examine alterations in protein expression following maternal immune activation. Poly(I:C) substantially reduced the major NMDA receptor subunit GluN1

and  $\alpha$ -synuclein in P21 animals, while no changes were seen in the embryos. These alterations may underlie cognitive and synaptic deficits (Forrest *et al.*, 2012, 2013, Khalil *et al.*, 2013, 2014) and provide an understanding of the molecular alterations induced by poly(I:C) and are likely to mediate some of the deficits in brain and behaviour reported previously (Meyer *et al.*, 2007). This suggests therapies aimed at correcting abnormalities in protein expression following maternal immune activation may prove fruitful in developing novel therapeutics to treat complex psychiatric disorders. Since glutamate receptors appear to be intimately involved in many of the effects of maternal immune activation, along with general neurodevelopmental processes, while also recognised as a major contributor towards schizophrenia, it was hypothesised that activation of the kynurenine pathway following infection may mediate a link between cytokine action and glutamate receptor dis-regulation contributing towards the increased incidence of neurodevelopmental disorders. Indeed, such a mechanism could account for, at least in part, the long-term effects of infection since studies show the dis-regulation of kynurenines are maintained for extended periods prolonging the effects of the initial infectious insult. For this reason, a parallel study within the laboratory examined the ability of maternal immune activation with poly(I:C) to activate the kynurenine pathway. Furthermore, since many CNS disorders during adulthood involve changes in kynurenine metabolites contributing towards disease progression (Giulian *et al.*, 1993; Pemberton *et al.*, 1997; Power and Johnson, 1995; Sei *et al.*, 1996; Heyes *et al.*, 1998), by mechanisms of neuroinflammation, it raises the possibility that changes in kynurenines early in life, or even prenatally by conditions affecting the pregnant mother, may affect cognitive and behavioural functions in the offspring later in life. Therefore, neurochemical changes in protein expression were examined following maternal disruption of the kynurenine pathway, to find selective delayed alterations. RhoB was reduced at P21 while Unc5H3 was reduced in the P60 cerebellum and may underlie the altered neuronal organisation frequently reported in schizophrenia. Furthermore, DCX was increased in the P60 hippocampus suggestive of an increase in postnatal hippocampal neurogenesis, while DISC-1 was increased in the P60 cortex and may be associated with behavioural and cognitive deficits. These changes in protein expression demonstrate the pathway to be highly active during development that may contribute towards the onset of neurodevelopmental disorders later in life.

### 1.1 Poly(I:C) Induced MCP-1 in Maternal Blood

The selective increased expression of chemokine monocyte chemoattractant protein-1 (MCP-1) in the maternal blood at 5 h post-injection with poly(I:C) confirms the efficacy of the selected dose to induce immune activation, while the absence in any change in IL-1 $\beta$  or TNF-

$\alpha$  levels confirms the transient nature of immune activation. Consistent with our findings, studies show maternal plasma levels of TNF- $\alpha$  are significantly elevated above control levels in as little as 2 h post-injection of pregnant rats with poly(I:C) at E16, while this significant increase returned to normal when examined at 8 h. This transient profile of TNF- $\alpha$  is confirmed by others where TNF- $\alpha$  was found to return to control levels by 4-6 h post-injection with poly(I:C) in mice (Cunningham *et al.*, 2007). Furthermore, poly(I:C) administration in mice increased plasma levels of IL-1 $\beta$  at 3 hours, while other groups, including ours, have failed to show an increase in this cytokine following poly(I:C) treatment (Fortier *et al.*, 2004; McLinden *et al.*, 2012). The lack of any change in other cytokines confirms no long-lasting or persistent inflammation that may confound the interpretation of the results, therefore, changes in protein expression reported here are in response to the acute maternal immune activation during gestation. Interestingly, there is a strong correlation between the levels of MCP-1 in the amniotic fluid and the subsequent appearance of autism spectrum disorders (Abdallah *et al.*, 2012), which may be related to the finding that MCP-1 can induce a cytokine expression profile resembling those observed in the disorder (Patterson, 2011). While the present study failed to measure the levels of inflammatory mediators in the maternal brain, several groups have shown the induction of both cytokines and chemokines following systemic injection of poly(I:C) in the pregnant mother. Intraperitoneal injections of poly(I:C) at comparable doses used in our experiments, have reported increased concentrations of chemokine genes including Cxcl9, Cxcl10, Cxcl11, Ccl2, Ccl7, Ccl5, and Ccl12 among others, including increased concentrations of the classical infection-induced cytokines (Konat *et al.*, 2009). Other studies show similar doses of poly(I:C) to increase the central expression of INF- $\alpha$ , INF- $\beta$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (McLinden *et al.*, 2012), while central microglial activation is also promoted by systemic treatment with poly(I:C).

#### *1.1.1 Changes in Foetal Brain Cytokine Levels Following poly(I:C) Treatment*

While our experiments failed to assess the levels of cytokines in the embryo brains, a large study examined whether prenatal infection with poly(I:C) in mice could induce changes in levels of cytokines within the foetal brain. This study demonstrated poly(I:C) exposure during gestation day E9 was associated with increased levels of pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , while poly(I:C) exposure during E17 increased IL-1 $\beta$  and IL-6. Interestingly, foetal brain expression of the anti-inflammatory cytokine IL-10 was decreased following poly(I:C) exposure at gestation day E9, but significantly increased following poly(I:C) exposure during E17 (Meyer *et al.*, 2006b), suggesting a loss in its ability to antagonise the actions of pro-inflammatory cytokines during earlier stages of gestation. Recent reports show similar elevations in levels of pro-inflammatory cytokines in foetal brains

following maternal immune activation with poly(I:C) (Abazyan *et al.*, 2010), however, a limitation applying to all embryo studies on foetal brains is that the analysis was performed on whole foetal brains, while changes in cytokine levels, and their effects on neuronal populations, may likely be region specific.

To address the question whether maternally produced cytokines undergo transplacental transport to affect foetal development, laboratories employed the use of injecting radiolabelled cytokines into the pregnant rodent. IL-6 was shown to undergo transplacental transport since radiolabelled IL-6 ( $^{125}\text{I}$ -IL-6) was detected in the foetus in as little as 30 min following injection in pregnant rats on E11-E13 and E17-E19 (Dahlgren *et al.*, 2006). Of interest, foetal levels of  $^{125}\text{I}$ -IL-6 were higher in embryos exposed to the radiolabelled cytokine in earlier stages of gestation (E11-13) when compared to later stages (E17-19). This demonstrates IL-6 can readily cross the placenta during early/middle gestation but not so easily during late gestation. Furthermore, while an *in vitro* preparation of human placenta at term has shown IL-6 to undergo transplacental transfer, minimal transfer of IL-1 $\beta$  and TNF- $\alpha$  was noted (Zaretsky *et al.*, 2004). The variability in transplacental transport of cytokines throughout gestation may be important in identifying critical time points of vulnerability to prenatal immune challenge. This shows maternally produced cytokines undergo transplacental transfer, gaining entry into the foetal compartment, while transplacental transfer of cytokines is at a maximal rate during earlier stages of gestation as opposed to later stages, a factor that may contribute to prenatal infection during early stages of gestation as being associated with greater brain and behavioural deficits including the greatest risk for schizophrenia (Brown *et al.*, 2004; Meyer *et al.*, 2007, 2006, 2009). Therefore, despite cytokine concentrations being elevated in the foetal brain, much evidence suggests maternally produced cytokines may be transported via the maternal circulation through an immune compromised blood placental barrier (BPB).

The placenta itself may constitute a second source of cytokines affecting the foetal system. Cells of the placental barrier, including trophoblasts, uterine epithelial cells and chorionic villi express a variety of TLRs (Abrahams and Mor, 2005; Krikun *et al.*, 2007). It is therefore likely, in the event of maternal infection; cells of the placental barrier may mount an additional cytokine response leading to altered levels of cytokines in the foetal compartment. Additionally, the foetus itself may produce and secrete its own cytokine proteins following maternal infection, however, this would largely depend upon the developmental stage of the foetus since a functional immune system requires a series of well-coordinated developmental events beginning early in foetal life. In most mammals during early or middle stages of



pregnancy the foetal immune system is relatively underdeveloped, while functional maturity is only achieved during late gestation and postnatal stages of development (Holsapple *et al.*, 2003). Therefore, the ability for the foetus to initiate its own cytokine response as a reaction to maternal infection would critically depend upon the gestational stage of the foetus.

### 1.1.2 *Direct Action of Virus*

Although the majority of studies demonstrate the deficits in behaviour during adulthood, caused by prenatal infections, are most likely mediated by indirect mechanisms due to altered balances of pro-inflammatory cytokines, there remain a few infectious pathogens that may induce neurodevelopmental deficits in the foetal brain by accessing the foetal environment and exerting a direct action on the foetal brain. While LPS cannot cross the placental barrier, it is widely established the rubella virus can readily cross the placental barrier and enter the foetal brain (Fatemi, 2005). Apart from activating specific pro-inflammatory cytokine reactions, the rubella virus can exert other consequential effects including inhibiting mitosis and myelination (Boue' and Boue', 1969). Specific strains of the influenza virus have also been shown to undergo transplacental transfer, where pregnant mice infected with the influenza A/WSN/33 strain enters the foetal brain and persists even at postnatal stages. Furthermore, the resulting offspring exhibit altered patterns of gene expression in the CNS (Asp *et al.*, 2005), indicating that the influenza A/WSN/33 strain effects on the offspring may also be a contributing factor towards the neuropathology observed during adulthood. On the contrary, maternal infection with the influenza A/NWS/33 strain does not cross the placenta and is not found in the foetal brain (Shi *et al.*, 2005), however, the resulting offspring exhibits pharmacological and behavioural deficits similar to schizophrenia during adulthood (Shi *et al.*, 2003). Firstly, this demonstrates the strain or type of infectious pathogen is important since not all cross the placental barrier, and secondly, even in the absence of a virus undergoing transplacental transfer, it is still capable of producing behavioural deficits in the offspring. It may be expected that infectious agents capable of both triggering the maternal immune response and crossing the placental barrier, to gain access to the foetal brain, may contribute to greater neurodevelopmental deficits precipitating in the offspring during adulthood. This may explain why the greatest risk of schizophrenia is observed with the rubella virus in the first trimester of pregnancy, since cytokine passage to the embryo is at a maximal rate, while the virus also enters the foetal compartment (Dahlgren *et al.*, 2006; Zaretsky *et al.*, 2004; Fatemi, 2005; Asp *et al.*, 2005).

### 1.1.3 Cytokine Actions via Sickness Behaviour

Another mechanism involves the induction of sickness behaviour, a behavioural complex typically observed following acute infections and tissue injury in many mammalian species, often resembling depressive-like symptoms. The behavioural characteristics often accompanied with sickness behaviour include behavioural and physiological changes in the host and in rodents. These effects are characterised by a fever response, malaise, reduced exploratory and social investigation and a decrease in food and water intake frequently leading to weight loss (Dantzer, 2001). There is evidence sickness behaviour is mediated through the effects of pro-inflammatory cytokines, including IL-1, TNF- $\alpha$  and IL-6 (Qin *et al.*, 2007; Dantzer *et al.*, 2008; Burton *et al.*, 2011). Furthermore, the effects of inflammation-induced anorexia is directly proportional to the severity of the initial insult (Lennie, 1998), a mechanism which may limit the intake of dietary iron, a nutrient employed by bacteria for bacterial growth, and dietary zinc. Pro-inflammatory cytokines mediate the reduction in the circulation of iron (Lee *et al.*, 2005), via a mechanism involving the pro-inflammatory cytokine IL-6 (Nemeth *et al.*, 2004). Although this inflammation-induced state of hypoferrremia is a protective mechanism limiting its availability to invading pathogens, iron is an essential nutrient for neurodevelopment where a reduction during several stages of development result in long-term alterations in dopaminergic neurotransmission (Kwik-Urbe *et al.*, 2000). Within the context of maternal infection, this may be relevant in contributing towards the prolonged actions of cytokines while providing a mechanism for affecting dopaminergic systems in the offspring.

Similarly, pregnant mice fed a high zinc (Zn) diet prevent deficits in object recognition in adult offspring when compared to control animals prenatally exposed to LPS alone (Coyle *et al.*, 2009), implying a decrease in maternal or foetal zinc levels induced by LPS. Cytokines induce the synthesis of metallothionein (MT), a zinc-binding protein thereby reducing circulating levels of zinc. Wild-type mice treated with LPS decrease circulating levels of zinc, an effect preceded by the induction of MT, whereas LPS treatment in MT-knockout mice produced no change (Carey *et al.*, 2003). Therefore, the infection-induced reduction of zinc in the mother and potentially the foetus, through a reduced maternal transport, results in developmental abnormalities in the offspring. Consistently, supplementation with higher levels of zinc in the maternal diet, normalising zinc levels in the CNS, protects against birth defects (Chau *et al.*, 2006). Immune-induced malnutrition observed during the sickness behaviour is important since several groups have demonstrated a positive association between prenatal malnutrition and the subsequent development of schizophrenia in the offspring during adulthood (Susser *et al.*, 1996; Brown *et al.*, 1996).

Interestingly, changes in feeding activity during maternal infection may also be due to alterations in kynurenines. The concentration of KYNA is significantly decreased in patients with anorexia while the concentration of QUIN is significantly increased (Demitrack *et al.*, 1995). QUIN reduces feeding behaviour via activation of NMDA receptors since the glycine-2-site agonist D-serine induces the same response (Sorrels and Bostock, 1992), while administration of the KYNA analogue, 7-chlorokynurenic acid, reverses this effect subsequently enhancing feeding behaviour. This suggests that brain concentrations of kynurenines, may be an important regulator of feeding activity, and may contribute towards the malnutrition observed during the sickness behaviour reported following LPS or poly(I:C) exposure.

## 1.2 Poly(I:C) Failed to Induce Activation of the Kynurenine Pathway

Pursuing the hypothesis that activation of the kynurenine pathway following poly(I:C) exposure in the pregnant mother may be a mechanism underlying the molecular alterations observed in the offspring, a parallel study within the laboratory assessed whether poly(I:C) activated the kynurenine pathway. Interestingly, in our animals, poly(I:C) failed to activate the kynurenine pathway and induced no changes in KYN or KNYA in the maternal blood or embryos (Khalil *et al.*, 2014), suggesting that changes in protein expression or general sickness behaviours are not dependent upon changes in kynurenines. In contrast to this are several groups showing infection with HIV or influenza or toxoplasma gondii induces activation of the kynurenine pathway in astrocytes and fibroblasts, while increasing IDO and TDO activity in mice and in human neuroepithelial cells (Fujigaki *et al.*, 2001; Fox *et al.*, 2013; Huang *et al.*, 2013; Xiao *et al.*, 2013). However, discrepancies in IDO activation following infection have also been reported (Niño-Castro *et al.*, 2013) suggesting it may be related to species and pathogen specificity. In support of this are reports that while viral infection with HIV-1 strain induced the activation of IDO and QUIN in human foetal brain tissue (Kerr *et al.*, 1997), and an increase in IDO and KYN in cultured human monocyte-derived macrophages when infected with HIV-1, HIV1-JRFL and HIV1-631 viral strains (Grant *et al.*, 2000), no changes were seen in IDO activation or kynurenine metabolites following infection with the HIV1-BaL strain. Since the ability of HIV-1 strain to activate the kynurenine pathway appears to be due to the conservation of key amino acids in the third hypervariable region (V3) of glycoprotein (gp)-120 (Korber *et al.*, 1994; Power *et al.*, 1994), and viruses are known to mutate and evolve, mutations in this region may underlie its loss in ability to activate the kynurenine pathway, and may account for why only 30% of AIDS patients later develop dementia involving kynurenine input.

*In vitro* studies show LPS also activates the kynurenine pathway in human foetal brain tissue accompanied with a rise in QUIN concentration (Kerr *et al.*, 1997), while recent reports extend this finding to whole blood cultures (Krause *et al.*, 2012). Recently, *in vivo* models of infection demonstrate that peripheral treatment with LPS results in the activation of IDO (O'Connor *et al.*, 2011; Walker *et al.*, 2012; Salazar *et al.*, 2012), and intracerebrovascular injection of LPS in mice also induces central activation of IDO with a rise in brain levels of KYN (Lawson *et al.*, 2013). Similarly, poly(I:C) administered to rats activated the kynurenine pathway and was associated with the induction of IDO expression and an increase in KYN (Gibney *et al.*, 2013). However, activation of the kynurenine pathway was noted at around 24 h post-treatment, while our assessment was restricted to 5 h post-injection. Furthermore, HIV-1 infection in an *in vitro* experiment also noted increased IDO and KYN synthesis after 48 h (Grant *et al.*, 2000), while an *in vivo* mouse model of early postnatal infection with the influenza virus during P3, showed activation of the kynurenine pathway during P7 and P13 (Holtze *et al.*, 2008). Together, these studies imply the activation of the kynurenine pathway is likely to constitute a more delayed response following exposure to infection, as opposed to an immediate effect. This suggests that while the kynurenine pathway is not involved in any changes in protein expression in the embryo brains at 5 h following prenatal administration of poly(I:C), alterations detected at P21 may involve changes in kynurenines, although this was not sufficiently addressed nor confirmed in our experiments.

### 1.3 Ro61-8048 Does Not Cross the Blood-Brain-Barrier

Despite several studies showing the KMO inhibitor Ro61-8048 to offer neuroprotection in a variety of *in vitro* and *in vivo* models of stroke, cerebral ischemia, epilepsy and Parkinson's disease, it is surprising Ro61-8048 is claimed to be impermeable to the BBB (Schwarcz *et al.*, 2009). Therefore, the central effects of Ro61-8048 achieved *in vivo* following oral administration (Rover *et al.*, 1997) are attributed to changes in kynurenine levels peripherally with subsequent changes occurring in the brain. Although Ro61-8048 cannot penetrate the BBB, the subsequent rise in KYN can readily cross the BBB where it is taken up by glial cells and converted into QUIN and KYNA. This is demonstrated experimentally where systemic administration of KYN increases KYNA in the brain (Miller *et al.*, 1992; Nozaki and Beal, 1992), illustrating that KYNA was increased as a direct result of KYN entering the CNS following systemic injection. Moreover, tryptophan, KYN and 3-HK readily cross the BBB due to simple diffusion across vascular membranes while they are also actively transported into the CNS via large neural amino acid transporters (Speciale and Schwarcz, 1990), leading to the elevation of these compounds, including QUIN, in the brain

(Juach *et al.*, 1993), presumably due to the further metabolism of KYN and 3-HK. In addition to the ability of endothelial transporters to increase kynurenine metabolites in the CNS, endothelial cells may also determine the amount of tryptophan metabolism and dictate the levels of kynurenine metabolites available for transport. One group showed KYN taken up by vascular endothelial cells was converted to KYNA via KAT (Wejksza *et al.*, 2004), and there is ample evidence suggesting changes in vascular kynurenine metabolites may play a role in modulating blood pressure (Wang *et al.*, 2010). Furthermore, since IDO activation in endothelial cells, in response to pro-inflammatory mediators, will increase kynurenine metabolites and alter local tryptophan levels, it is likely that these changes will induce changes in CNS tryptophan and kynurenine levels, especially since studies suggest that the kynurenine synthesised is secreted primarily from the basolateral pole of the endothelial cells, thereby gaining direct access to the cerebral aspect of the BBB (Owe-Young *et al.*, 2008). This may particularly be important in the context of immune activation where the integrity of the BBB is compromised, permitting many compounds not normally penetrable, including QUIN and KYNA, entry into the CNS.

### 1.3.1 Ro61-8048 Increased Central Kynurenine and Kynurenic Acid

While events may be restricted to the periphery, changes in tryptophan/kynurenine ratios in the blood by IDO or TDO can produce marked secondary changes in the concentration of kynurenine metabolites in the CNS. This was confirmed for our experiments in a parallel study where HPLC analysis examined changes in kynurenines, to reveal an intraperitoneal injection of Ro61-8048 in the pregnant dam substantially increased the levels of KYN and KYNA in the maternal plasma and brain at 5 h, while these changes were mirrored in the embryo brains. Maternal levels of KYN and KYNA in the blood and brain returned to control values at 24 h, while they remained substantially elevated in the embryo brains (Forrest *et al.*, 2013). This confirmed the ability of Ro61-8048 to inhibit KMO in our experiments, and that changes seen in animals born to mothers treated with Ro61-8048 are due to alterations in kynurenine metabolites, since to date, Ro61-8048 is reported to be a selective KMO inhibitor while other actions of the drug have not been identified. Interestingly, Ro61-8048, while increasing KYNA levels appeared to produce no effect on the levels of QUIN. This may be due to compensatory mechanisms increasing enzyme expression and activity, or due to the synthesis of QUIN via an alternative route. Since KYN can be metabolised to anthranilic acid then oxidised to produce 3-hydroxyanthranilic acid (3-HAA) and converted to QUIN, this may account for the lack of change in QUIN levels following KMO inhibition. Indeed, this route of QUIN production is increasingly becoming recognised as being recruited in a range of inflammatory conditions where levels of anthranilic

acid are substantially increased (Darlington *et al.*, 2010). Furthermore, the lack of change in QUIN demonstrates that deficits observed in our experiments following Ro61-8048 treatment in rats and those reported in alleviating symptoms in a variety of CNS disorders, are mediated by the rise in KYNA as opposed to a reduction in QUIN.

## 1.4 Discussion of Results

### 1.4.1 *Poly(I:C) Decreases GluN1 Expression*

Schizophrenia has long been attributed to abnormal alterations in dopaminergic signalling within the mesolimbic cortex, however, the dopamine hypothesis for schizophrenia has failed to account for all the symptoms observed. The first line of evidence involving a reduced activity state of glutaminergic transmission was provided when low levels of glutamate were found in the cerebrospinal fluid (CSF) from schizophrenia patients (Kim *et al.*, 1980). This provided a shift in the way schizophrenia was viewed, ultimately proposing a hypofunction of the glutamate transmitter system may contribute to the symptoms of schizophrenia. This was strengthened by later findings that pharmacological blockade of NMDA receptors by phencyclidine (PCP) and ketamine in both animal models and humans, were capable of inducing profound symptoms reminiscent of those observed in schizophrenia (Javitt & Zukin, 1991; Krystal *et al.*, 1994), thereby implicating disturbances in NMDA receptor activity with the emergence of psychiatric symptoms. Interestingly, neureglin-1 (NGR1), a schizophrenia susceptibility gene, and its receptor erB, is implicated in the pathophysiology of schizophrenia where its stimulation suppresses NMDA receptor activation in the human prefrontal cortex as examined in post-mortem tissue. Since schizophrenia is accompanied by enhanced NRG1-erB signalling, it is proposed this may be a mechanism contributing towards NMDA receptor hypofunction. The reduced activity of NMDA receptors during schizophrenia is now a well-established phenomenon, and forms an interesting parallel to this study where GluN1 was down-regulated in P21 groups of animals born to mothers treated with poly(I:C). The duration of GluN1 down-regulation in these experiments is unknown, but since no change was seen in the embryos at 5 h, it may represent a more delayed effect of poly(I:C). GluN1 is encoded by a single gene while differential splicing of three exons can generate up to eight GluN1 splice variants producing molecularly diverse forms of the NMDA receptor, presumably contributing towards their wide range of properties in different neuronal populations (Zukin and Bennett, 1995). However, the antibody elected for use in this study detects all known splice variants; therefore, the reduction of GluN1 reflects an overall change of NMDA receptor number or function.



Consistent with our finding, one group showed a reduction in the transcript expression of GluN1 receptor subunit in the thalamus of schizophrenia patients that was limited to exon 22-containing isoforms only, and did not involve exon 5 or exon 21-containing isoforms (Clinton *et al.*, 2003). Another independent study reported a 30% reduction in GluN1 mRNA expression in tissue homogenates of the superior temporal cortex of patients with schizophrenia who exhibited cognitive impairments (Humphries *et al.*, 1996). Furthermore, a 30% reduction of hippocampal expression of GluN1 was enough to produce deficits in sensorimotor gating while spatial memory remained unaffected (Inada *et al.*, 2003). Consistently, a mouse model engineered to express low levels of GluN1 subunit also displayed deficits in sensorimotor gating as examined in the paradigm of prepulse inhibition (Duncan *et al.*, 2004). The GluN1 receptor subunit is crucial for every functional NMDA receptor, and GluN1-knockout studies in mice prove fatal ultimately leading to death around 8-15 h after birth (Forrest *et al.*, 1994).

Animal models of prenatal infection have also reported neuropathological effects that resemble some of the neuroanatomical and neurochemical deficits associated with schizophrenia. Prenatal exposure to poly(I:C) during E9 reduced GluR1-IR in the NAc shell of female adult offspring, while no change was observed in GluN1-IR (Meyer *et al.*, 2008c). It is likely no change was found in the immunoreactivity of GluN1 since it was examined in a highly localised region of the brain, while our experiments examined the entire half of the cerebral hemisphere to find an overall reduction in GluN1. Additionally, this group employed a single poly(I:C) injection during E9 which differs from the triple injection schedule on E14, 16 and 18 employed in our experiments. Poly(I:C) disrupts GABAergic markers in the hippocampus and prefrontal cortex, while a significant reduction is found in the expression of GluN1 in the hippocampus (Meyer *et al.*, 2008b), resembling the abnormalities of human schizophrenia (Hashimoto *et al.*, 2008; Gao *et al.*, 2000). Similar work on neonatal inflammation shows LPS exposure at P5, P30 and P77 reduces mRNA levels of GluN1 in the hippocampus of adult rats accompanied by behavioural deficits (Harre *et al.*, 2008).

mRNA expression of the GluN1 subunit is present at low levels during embryonic day 14 and gradually increases during development. A substantial increase is observed in the cerebral cortex, hippocampus and cerebellum between postnatal days 7 and P20 (Zhong *et al.*, 1995), after which there is a slight decline to adult levels (Laurie and Seeburg, 1994). The time period at which GluN1 was significantly reduced in our experiments corresponds directly with the end of the developmental increase that normally occurs, therefore, it may be that an absence in this increase during development, in response to poly(I:C), may be equally as

important and pharmacologically as relevant as the effects of an antagonist blocking the receptor during this period. Indeed, MK-801-treated rats on P7 exhibit reduced neuronal numbers and volume within the hippocampus, including an altered expression of GluN1 during adulthood accompanied with impairments in PPI and increased locomotor activity during adolescence and early adulthood (Harris *et al.*, 2003; Uehara *et al.*, 2009). Therefore, the present finding of a reduction in the overall number or function of NMDA receptors, during a period that should be accompanied by a developmental rise, may contribute towards some behavioural abnormalities during adulthood in response to poly(I:C).

Within a developmental context, since neurogenesis and synaptogenesis crucially depend upon the activity of NMDA receptors, these processes are sensitive to disturbances in NMDA function, where alterations can induce excitotoxic and apoptotic neurodegeneration (reviewed in Olney, 2002). This is especially important in the developing brain with respect to the growth spurt that is known to occur and varies between different brain regions, species examined and trajectory timescales. In rodents, it occurs from around birth until two-three weeks after birth, while in humans the growth spurt exists during the last three months of gestation until around three years after birth (reviewed in Dobbing, 1974; Rice & Barone, 2000). A reduction in NMDA receptor number or function during this period is critical since the pharmacological blockade of NMDA receptors in neonatal rodents induces massive neuronal loss and apoptotic neurodegeneration (Ikonomidou *et al.*, 1999) with brain and behavioural deficits becoming evident during adulthood (Harris *et al.*, 2003, Kawabe *et al.*, 2007; Kawabe & Miyamoto, 2008), many of which strikingly resemble the abnormalities occurring in schizophrenia. Alterations in GluN1 here may induce substantial abnormalities in neuronal integrity and synaptogenesis, since NMDA receptors are involved in all aspects of neuronal development. Although GluN1 did not change in the embryos, a parallel study in the laboratory found a significant increase in protein expression of the NMDA receptor subunit GluN2B which is likely to perturb NMDA function since different subtypes confer different properties upon the receptor. Interestingly, an increase in GluN2B expression is reported in schizophrenia patients, and contributes towards cognitive deficits (Baraldi *et al.*, 2002). Furthermore, there is a critical period of programmed cell death that occurs during development up until the third year of life, which shapes the nervous system by inducing apoptosis to eliminate a large proportion of neurons. Therefore, an induced change in NMDA receptor function during this period of development could be involved in either accelerating or slowing, amplifying or suppressing the rate of developmental apoptosis. These studies illustrate the essential role of NMDA receptors and demonstrate that activation of the immune system can interfere with their normal functioning during brain development, and



may account for some of the neurochemical and behavioural abnormalities reported following maternal infection.

#### 1.4.2 *Ro61-8048 Induces no Change in GluN1 and PSD-95 Expression*

Owing to the crucial link between NMDA receptors and schizophrenia, the expression of GluN1 was examined following prenatal disruption of the kynurenines. Since KMO inhibitors produce changes in kynurenine levels thereby regulating NMDA receptors, it was expected that prenatal administration of Ro61-8048 may alter the protein expression of GluN1, however, no significant changes were seen in the embryo brains at 5 or 24 h post-treatment. Similarly, when compared with the protein expression in the P21 animals, GluN1 protein expression remained comparable with control animals. Since the full blown spectrum of schizophrenia symptoms become apparent during early adulthood, GluN1 protein expression was examined in P60 animals in the hippocampus, cortex, midbrain and cerebellum. Similarly, no significant changes were noted in any of these regions, suggesting prenatal inhibition of KMO does not affect the total number of NMDA receptors.

PSD-95 was examined since it can regulate the activity of NMDA receptors. PSD-95 is a large protein complex containing PDZ (Postsynaptic density-95, Discs large, and Zonula occludens-1) binding domains located on postsynaptic sites where NMDA receptors are clustered and anchored to postsynaptic membrane via the C-terminal of GluN2 subunits (Ziff, 1997; Cousins *et al.*, 2008). PSD-95 localise and form clusters at synapses early during synapse formation, regulating the recruitment of many proteins to the macromolecular complex (Morabito *et al.*, 2004), contributing towards synapse development, maturation (El-Husseini *et al.*, 2000; Chetkovich *et al.*, 2002) and plasticity (Guan *et al.*, 1996). Apart from PSD-95 serving a scaffolding role for NMDA receptors, it is an essential regulator of ionotropic glutamatergic neuronal signalling (Schluter *et al.*, 2006; Xu *et al.*, 2008), and also involved in the trafficking and internalisation of receptor complexes. Although changes in PSD-95 may be linked with depression (Toro and Deakin, 2005; Kristiansen and Meador-Woodruff, 2005; Beneyto and Meador-Woodruff, 2008) and KYNA is elevated during depression, no changes were seen in the protein expression of PSD-95.

The concept of prenatal changes in kynurenines altering the function of NMDA receptors in the offspring was confirmed in a parallel study which examined the relative protein expression of the NMDA receptor subunits, GluN2A and GluN2B. Interestingly, when examined in the embryo brains at 5 h, Ro61-8048 induced highly selective alterations in opposite directions, while other proteins specific to neurodevelopment remained unaffected.

This suggests Ro61-8048 does not produce immediate deficits in neurodevelopmental processes like neurogenesis, neurite outgrowth and axonal guidance, but selectively alters the function of NMDA receptors. Embryo brains at 5 h were characterised by a reduction in GluN2A protein expression, while GluN2B was substantially increased thereby altering their relative ratios. Usually GluN2B mRNA levels are highest at P1 while declining to undetectable levels around P28 (Zhong *et al.*, 1995), while GluN2A rises rapidly around early development reaching adult levels by P22 (Wang *et al.*, 1995). This phenomenon, referred to as the developmental switch, is thought to confer different properties to NMDA receptors during development and adulthood. GluN2A and GluN2B are implicated in a range of CNS functions including learning and plasticity (Lemay-Clermont *et al.*, 2011; Rammes *et al.*, 2009), while the relative ratios between them are proposed as central to some aspects of brain development, neuronal function (Brightman *et al.*, 2010; McKay *et al.*, 2012), cell viability, degeneration and protection (Hardingham and Bading, 2010; Vasuta *et al.*, 2007; Hu *et al.*, 2009). Therefore, Ro61-8048 alters the developmental ontogenic profiles of these subunits which could prove deleterious to early postnatal development. These alterations would significantly perturb the function of NMDA receptor during critical periods of brain development, and may be related to changes in behaviour and synaptic plasticity during adulthood (Traynelis *et al.*, 2010). Interestingly, changes in GluN2A and GluN2B had normalised by 24 h, suggesting rapid compensatory physiological adaptations, however, alterations in these subunits also became apparent in P21 and P60 animals, suggesting these physiological adaptations are poorly maintained. GluN2B and GluN2A were both significantly increased in P21 animals, while only GluN2A remained affected in the hippocampus and cortex of P60 animals. The increase in GluN2A following Ro61-8048 treatment may be related to schizophrenia, since elevations in GluN2A protein transcripts are reported in the dorsolateral prefrontal cortex and the occipital cortex of patients with schizophrenia (Dracheva *et al.*, 2001). GluN2A is also important in LTP, where an increase induces LTP (Baez *et al.*, 2013), therefore, the reduced expression of GluN2A in the P60 hippocampus is consistent with reduced LTP noted in these animals from a parallel study. The role of GluN2B in cognition and plasticity has also been confirmed in mice overexpressing GluN2B where animals display alterations within these parameters (Cui *et al.*, 2011). Although our experiments show GluN2B levels normalise by P60, since it is considerably important in cerebral development, the early embryonic and postnatal changes in its expression may have permanently altered the sequence of events leading to new neuronal formation.

### 1.4.3 DISC-1 Expression Remains Unaffected by poly(I:C) Treatment

DISC-1 is important in neuronal development during embryogenesis and is identified as a candidate gene for schizophrenia. Transient knockdown of DISC-1 in mice, during prenatal development, produces dopaminergic and behavioural deficits in the adult offspring (Niwa *et al.*, 2010). Therefore, it is entirely possible that if genes like DISC-1 were to be dysregulated, even for a short period during prenatal development, it could potentially account for a mechanism by which prenatal infection induces long-term changes in the adult CNS by causing disruptions in dopaminergic systems. While a plausible theory, since no significant changes were found in the embryos or at P21, it suggests that abnormalities in the dopaminergic system and behaviour, reported from other studies, do not involve the altered expression of DISC-1 during prenatal development. Although it remains possible that environment-immune interactions may respond differently during the first or second trimester of pregnancy, since the strongest association of DISC-1 with schizophrenia appears to be largely genetic, as opposed to environmental, it seems unlikely DISC-1 is involved. Environmental interactions only appear to be relevant in the presence of mutant forms of DISC-1 genes, resembling those involved in schizophrenia. In support of this, a significant interaction is reported where prenatal infection with poly(I:C) during E9 interacted with a mutant form of DISC-1 (mhDISC-1) producing elevated anxiety levels, depression-like responses, altered patterns of sociability and attenuated reactivity to stress (Abazyan *et al.*, 2010). Interestingly, behavioural abnormalities were dependent upon the continuous expression of mhDISC-1, suggesting the reversibility of such effects. Another study showed prenatal exposure to poly(I:C) in mice genetically modified to exhibit a point mutation in the DISC-1 gene, were more sensitive to the effects of maternal immune activation than wild-type mice (Lipina *et al.*, 2013). Similarly, mice neonates constitutively expressing mutant DISC-1 also reported behavioural abnormalities including deficits in object recognition and fear conditioning when treated with poly(I:C) (Ibi *et al.*, 2010). These studies demonstrate significant environment-gene interactions in the presence of mutant forms of DISC-1 genes, likely to resemble conditions of immune challenge in individuals possessing schizophrenia susceptibility genes like DISC-1. The presence of susceptibility genes offer an explanation as to why most humans exposed to infection alone fail to develop schizophrenia during adulthood.

The relationship between NMDA receptors and DISC-1 has also been examined, focussing on the effects on synaptic density as a consequence to NMDA hypofunction in a genetic mouse model in which levels of GluN1 was reduced by 10%. These experiments reported a reduction in synapse number in an age-dependent manner observed at the post-

pubertal age of 6 weeks. Interestingly, in an attempt to identify the biochemical basis of this effect, synapse-specific reductions in the protein expression of DISC-1 were found. Furthermore, the pharmacological blockade of the NMDA receptor with MK-801 produced a similar reduction in both spine density and DISC-1, indicating that levels of DISC-1 are regulated by NMDA receptor function (Ramsey *et al.*, 2011). However, since our experiments showed that although there was a significant reduction in the major NMDA receptor subunit GluN1, no change was seen in DISC-1 suggesting that DISC-1 is not dependent upon the expression of GluN1.

#### *1.4.4 Ro61-8084 Increases DISC-1 Expression in the Cortex of P60 Animals*

Accumulating evidence from genetic studies have linked DISC-1 not only with schizophrenia and depression, but also other psychiatric disorders of neurodevelopmental origin including depression, autism and Asperger syndrome (Kilpinen *et al.*, 2008; Song *et al.*, 2008, 2010; Osebun *et al.*, 2011). Since the initial hypothesis remained that changes in kynurenines during development may affect neurological outcome in the offspring leading to CNS disorders, we sought to examine the expression of DISC-1 following Ro61-8048 treatment. Furthermore, as changes in kynurenines regulate NMDA receptors, and a parallel study showed changes in NMDA receptor subunits, it seemed appropriate to examine its effect upon DISC-1 expression. DISC-1 plays a critical role in regulating cellular proliferation in the developing cerebral cortex (Mao *et al.*, 2009), however, when examined in the embryo brains at 5 and 24 h, no significant changes were noted. Therefore, although NMDA receptor function is greatly altered, DISC-1 is not involved in inducing abnormalities during embryogenesis and cellular proliferation in the developing brain controlled by DISC-1 occurred normally. These findings were extended with no changes observed in P21 animals, further confirming the lack of developmental abnormalities produced by DISC-1, however, Ro61-8048 produced a delayed increase in the expression of DISC-1 in the P60 cortex. Since changes in DISC-1 protein expression were only observed in young adult rats, this shows while DISC-1 does not influence development, it may well affect multiple cellular processes after brain development thereby influencing brain maturational processes (Chubb *et al.*, 2008; Brandon and Sawa, 2011). The prime location of DISC-1 also appears suited for a prominent role in schizophrenia pathology. While nuclear, cytoplasmic and actin-associated locations are evident, DISC-1 expression is predominantly expressed in mitochondria (James *et al.*, 2004), which is of interest since several mitochondrial deficits are found in schizophrenia

Neurogenesis in the adult brain is restricted to two primary locations, in the subgranular zone of the dentate gyrus and the subventricular zone of the cerebellum. In these

regions, developing neurons are guided largely along pre-existing neuronal networks migrating to limited areas of the cortex. Neuronal migration is a fundamental cellular process required for optimal cortical organisation; therefore the significant impairment observed in the cortex suggests DISC-1 may be involved in inducing deficits in neuronal migration of newly developing neurons. This is supported by consistent reports showing alterations in DISC-1 function, produce deficits in neuronal migration, aberrant integration and misfiring (Kubo *et al.*, 2010; Singh *et al.*, 2010; Ishizuka *et al.*, 2011). Some disruptions to DISC-1 appear to alter the rate and speed of neuronal migration substantially thereby producing aberrant synaptic connections. While the functional activity of these synaptic connections is unknown, this may produce incorrect neuronal connections thereby contributing to the ‘mis-wiring’ of synaptic connections observed in schizophrenia. Although current evidence links early development to schizophrenia, the function and dis-regulation of adult-born neurons in the pathophysiology of schizophrenia is an intriguing new hypothesis. Intriguingly, these neuronal processes mediated by DISC-1 involve GTPase-dependent signalling mechanisms (Chen *et al.*, 2011), and since RhoB was significantly down-regulated in the P21 animals, this may further induce deficits in neuronal migration.

The finding that prenatal modification of the kynurenine pathway during development regulates the expression of DISC-1 is one of the major findings providing a mechanism by which elevation of KYNA during development may serve to increase the incidence of schizophrenia. Indeed, the substantial elevation of DISC-1 protein expression in the P60 cortex would be predictive of a substantial loss of AKT function in the cortex of these animals, a very consistent finding in schizophrenia. KIAA1212, a girdin protein regulating the actin cytoskeleton, binds to and increases AKT signalling activity in the absence of DISC-1, while in its presence, DISC-1 binds to its effector protein KIAA1212 thereby reducing AKT activity (Kim *et al.*, 2009b). Therefore, the substantial increase in DISC-1 measured in the cortex would be associated with a substantial loss of AKT activity. Consistently, this signalling pathway is reduced in post-mortem brains of schizophrenia patients (Arguello and Gogos, 2008), and is also considered a risk factor for the disease. Rodent models of maternal immune activation provide evidence of reduced AKT signalling, while mechanisms to suppress pro-inflammatory cytokines alleviate schizophrenia symptoms (Smith *et al.*, 2007; Cui *et al.*, 2009). It is therefore interesting to note that while rapamycin enhances AKT signalling, it is also a potent immunosuppressant, suggesting neuroinflammation may contribute towards the down-regulation of AKT following maternal immune activation. The cognitive and behavioural abnormalities precipitated by alterations in DISC-1 (Kvajo *et al.*, 2008), resembling cognitive deficits observed during schizophrenia, may be related to the loss of AKT function.

*Akt1* knockout mice display behavioural deficits associated with schizophrenia, including impaired prepulse inhibition of the startle response. Reduced AKT signalling is also associated with deficits in synaptic plasticity and cognitive behaviour, while brief treatment with rapamycin rescued these deficits in a mouse model of tuberous sclerosis (Ehninger *et al.*, 2008). Clinical data may also lend support to the theory that AKT enhancement is associated with increased neurological outcomes during schizophrenia, since typical and atypical antipsychotics, in addition to targeting specific receptor systems, also enhances AKT signalling.

While there is considerable evidence associating heightened levels of KYNA during schizophrenia, given that prenatal modification of central kynurenines induces significant impairments in the protein expression of DISC-1, a prime candidate gene for schizophrenia, this suggests the kynurenine pathway may be involved in the initiation of schizophrenia, as opposed to representing secondary changes to the disease. While current research suggests the increase in KYNA is associated with the reduced activity of NMDA receptors, thereby relating kynurenine levels with schizophrenia, the present data clearly demonstrates that elevations in KYNA can induce changes in gene expression of candidate genes for schizophrenia thereby accounting for the increase in their protein expression. DISC-1 represents one of these genes that can be regulated by KYNA. While schizophrenia possesses a maturation delay in the disease onset where the full blown spectrum of symptoms only emerges after post-pubertal maturity until early adulthood (Weinberger, 1987), intriguingly, changes in DISC-1 appear to coincide with this since no change was seen in adolescent P21 animals, and only became apparent during early adulthood, in P60 animals. This further emphasises a potential developmental role for the kynurenine pathway, while demonstrating the pathway can mirror the maturational delay seen in the disorder. Together, this lends support to extend the observations of KYNA in schizophrenia, to suggest changes in kynurenines prenatally may indeed serve a developmental model for schizophrenia. Furthermore, since a variety of environmental risk factors can affect the pregnant mother to induce changes in central kynurenines, the kynurenine pathway may represent a convergent intracellular pathway linking environmental interactions with the subsequent risk in schizophrenia.

#### *1.4.5 Poly(I:C) Administration is not Associated with RhoB Activation*

RhoGTPase enzymes RhoA and RhoB have consistently been associated with changes in synaptic plasticity and many aspects of neuronal development (Koh, 2006; Chen *et al.*, 2007) and neurogenesis, including being major contributing factors in disease. Rho proteins and



signalling pathways are intimately involved in processes including: axon growth cone dynamics, neurite outgrowth and extension, dendritic arborisation, cell morphology, cell division, membrane trafficking and cancers (Esteve *et al.*, 1998; Chaung *et al.*, 1997; Charest and Firtel, 2007; Jaffe and Hall, 2005; Ellis and Mellor, 2000; Ridley, 2006; Huang *et al.*, 2007). Since these enzymes exert many of their actions via regulation of the cytoskeleton to modify neuronal structure and function, they are often found to mediate actions of the NMDA receptor. However, although poly(I:C) reduced the expression of GluN1, no changes were seen in Rho proteins in the embryo brains or in P21 animals, suggesting they do not contribute towards brain and behavioural deficits following prenatal immune challenge.

Resting levels of RhoB remain low and are regulated in response to a variety of growth and stress signals including: epidermal growth factor (EGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), platelet-derived growth factor (PDGF), UV irradiation and genotoxic stress. (Cox and Der, 1997; Fritz and Kaina, 1997; Fritz *et al.*, 1995; Lebowitz *et al.*, 1997). RhoB plays a vital role in the signal transduction of damaged neurons where its expression is dramatically increased following CNS trauma, to selectively mediate apoptosis via activation of caspase-3 (Lakhani *et al.*, 2006). Indeed, Rho signalling following spinal cord injury contributes towards the inability of the adult CNS to regenerate by inducing neurite retraction and apoptosis, while inhibition of RhoA and RhoB or its downstream target Rho-associated kinase (ROCK), promotes growth of neurites and injured axons *in vivo* (Dergham *et al.*, 2002). These findings have been extended to models of focal cerebral infarctions and ischemia, associated with the activation of RhoB thereby resulting in widespread neuronal death, restricted to the infarct and ischemic hemisphere (Brabeck *et al.*, 2003). Similarly, in a murine model of stroke, up-regulation of RhoB was observed preceding the apoptosis of hippocampal neurons. Clinical studies also provide evidence that Rho proteins may have a role in mediating brain damage following stroke, since statins not only reduce cholesterol and low-density lipoprotein (LDL) levels in stroke patients, but are also potent inhibitors of RhoGTPases, raising the possibility that statin-induced neuroprotection may be mediated in part via inhibition of RhoB (Hess *et al.*, 2000). Collectively, these studies recognise RhoB as an early predictor of cell death, prompting intense interest in developing RhoB inhibitors as promising targets for limiting neuronal apoptosis following CNS trauma, with some compounds having entered clinical trials (Appels *et al.*, 2005). Models of CNS trauma conclude damaged neurons in a noxious environment of neuroinflammation with the induction of cytokines, chemokines, nitric oxide and oxidative stress, triggers the rapid induction of RhoB, therefore, it is surprising RhoB did not change in the embryo brains following poly(I:C) treatment in our experiments. It is likely since no actual mechanism of CNS trauma or neuronal damage was present, poly(I:C)

injection did not trigger an inflammation-induced RhoB induction. This is consistent with studies showing RhoB induction is largely dependent upon cell cycle arrest of damaged neurons, where RhoB inhibits Cyclin B1 (Kamasani *et al.*, 2004), a regulatory protein involved in mitosis, to induce apoptosis via JNK signalling pathways (Kim *et al.*, 2009a) and P53-dependent mechanisms, as opposed to responding to mere activation of toll like receptors by a synthetic compound. Although the expression of RhoA and RhoB did not change in our experiments, it is likely other GTPases, including Rac1 and Cdc42, may be involved in mediating some of the effects of maternal infection. This is supported by *in vitro* studies demonstrating TNF- $\alpha$ -induced reduction in neurite outgrowth and branching of hippocampal neurons involved RhoGTPase-dependent mechanisms (Neumann *et al.*, 2002).

#### 1.4.6 Ro61-8048 Down-Regulates RhoB in Postnatal Day 21 Animals

Despite a lack of change in RhoB in animals prenatally treated with poly(I:C), prenatal administration of Ro61-8048 significantly down-regulated its protein expression in P21 animals. This may represent a delayed change since RhoB expression measured in embryo brains at 5 and 24 h were comparable with controls. Cognitive function is dependent upon normal neuronal development, and since Rho proteins are involved in many aspects of these processes, the normal functioning of RhoGTPases are essential for normal cognitive development. There appears to be an intricate relationship between different members of the GTPase superfamily in regulating neuronal development, since Rac1 and Cdc42 promote dendritic growth and dynamics, while RhoA and RhoB inhibit dendritic growth and remodelling (Li *et al.*, 2000; Wong *et al.*, 2000). Axon growth cones respond to a variety of signals before extending their projections during neural development, and depend upon the relative ratio of these GTPases in determining dendritic growth, length and maturity. This suggests a reduction in RhoB during early postnatal development would be associated with a loss of inhibitory function upon dendritic growth and result in an overgrowth of dendrites and their projections. This is supported by genetic analysis where null mutations in Rho proteins in mosaic *Drosophila* brains resulted in neurons with over-extended dendrites (Lee *et al.*, 2000). Along with Rho proteins affecting dendritic arborisation, they are also important regulators of spine morphology, all of which contribute towards cognitive development. Collectively, the loss of RhoB may contribute towards the effects observed in disorders such as mental retardation, which is characterised by a reduction in spine density with abundance of long thin immature spines, at the expense of mature mushroom-like spines (Ramakers, 2002; Newey *et al.*, 2004). This is supported by the finding that many genes mutated in non-specific X-linked mental retardation (XMR) are indeed components of RhoGTPase signalling cascades (Linseman and Loucks, 2008).



Interestingly, these deficits related to a defective Rho signalling appear to resemble the reductions in dendritic arborisation and spine density (Diaz-Cintra *et al.*, 1990) induced by protein deprivation. Since protein deprivation is a form of malnutrition, this forms an interesting parallel as maternal malnutrition is linked with an increased incidence of neurodevelopmental disorders, and lends support to the hypothesis that Ro61-8048-induced down-regulation of RhoB may precipitate deficits in neuronal morphology relevant to developmental disorders. This is further strengthened by a neurodevelopmental model of schizophrenia, where maternal exposure to LPS in rats decreases dendritic length, arborisation and spine density in the hippocampus and medial prefrontal cortex at various postnatal ages (Baharnoori *et al.*, 2009), while prenatal infection with poly(I:C) reduces myelin thickness with a reduction in the immunostaining of myelin basic protein in the hippocampus (Makinodan *et al.*, 2008). Interestingly, these effects can also be produced by deficits in Rho signalling, since the loss of Rho proteins, including RhoB, induces abnormalities in hippocampal pyramidal cells in primary cultures (Linseman and Loucks, 2008), and evidence implicates Rho proteins in the formation of myelin sheaths (Angelica *et al.*, 2007), since oligodendrocytes and schwann cells lacking Rho proteins display defective thinner myelin sheaths coating axons (Benninger *et al.*, 2007). Since the pathology of schizophrenia includes deficits in hippocampal myelin including neuronal morphology, these studies lend support to the hypothesis that prenatal disruption of the kynurenine pathway may be important in the subsequent development of schizophrenia, and that a reduction in RhoB may precipitate morphological abnormalities within the hippocampus. However, despite the well-known roles of Rho proteins in cognition and cellular hippocampal morphology, changes in RhoB have not been reported in schizophrenia.

As dendritic spines form the major site of excitatory input to cells, and spine shape and function are intricately linked (Yuste *et al.*, 2000), abnormalities in spine density and shape are likely to produce impairments in synaptic transmission and plasticity. Indeed, RhoA and RhoB are involved in synaptic plasticity following activation of glutamate receptors. While RhoA suppresses synaptic plasticity, RhoB enhances the extent of neuronal plasticity in response to activation of NMDA receptors to induce long-term potentiation (LTP; O'Kane *et al.*, 2003). Consistent with the role of RhoB involved in mediating LTP in response to NMDA receptor activation, inhibition of its downstream signalling target ROCK, modifies the induction of LTP. Therefore, the decrease in RhoB at P21 observed here may be involved in mediating a reduction in LTP following NMDA receptor activation, which may also contribute towards deficits in plasticity observed in schizophrenia.

The role of Rho proteins appear to be of considerable importance within cancers where overexpression of RhoA, RhoC, Rac1 and Cdc42 result in their constitutive activation thereby rendering them insensitive to regulatory signals (Vega *et al.*, 2008; Wu *et al.*, 2004). This ultimately results in reduced cell apoptosis while promoting cell growth, survival and malignant cell transformation, invasion and metastasis leading to tumour development and progression (Puitt and Der, 2001; Ellenbroek and Collard, 2007). Since overexpressed Ras proteins are found in 30% of all cancers, farnesyltransferase inhibitors (FTIs) were developed as anti-cancer drugs to exploit the farnesylation requirement of Ras for its oncogenic activity. Surprisingly, FTIs were found to inhibit malignant cell growth and induce apoptosis in tumour cells via the inhibition of farnesylated-RhoB (RhoB-F), which subsequently increased the geranylgeranylated-RhoB (RhoB-GG) isoform thereby selectively inducing apoptosis of cancer cells while decreasing tumour progression (Prendergast, 2000). Furthermore, studies in transformed murine fibroblasts and in human carcinoma cells show enhancing the levels of RhoB-GG is sufficient in mediating the effects of FTI treatment (Du *et al.*, 1999; Du and Prendergast, 1999). The role of RhoB in cancers was confirmed with the finding that RhoB expression is dramatically down-regulated during cancers, often mediated by increases in other Ras proteins via phosphatidylinositol 3-kinase (PI3K) and Akt-dependent mechanisms. While this down-regulation of RhoB correlates well with cancer progression, ectopic expression of RhoB inhibits Ras, PI3K and Akt-mediated malignant transformation (Jiang *et al.*, 2004). Collectively, these studies suggest Ras-related cancers are largely due to the loss of RhoB-GG as opposed to the increase of other Rho proteins as demonstrated in a variety of cancer cell lines (Mazieres *et al.*, 2004; Sato *et al.*, 2007; Mazieres *et al.*, 2007). Therefore, considering the significant involvement of the kynurenine pathway during cancer, it is not surprising that prenatal disruption of this pathway produces changes in RhoB that may contribute towards the onset or progression of cancers in susceptible individuals already vulnerable to the disease due to health, environmental or genetic predispositions.

Interestingly, the loss of RhoB seen in our experiments appears to be important only at P21 since at P60, RhoB levels return to normal. While this suggests no further deficits induced by RhoB, it is likely to have produced several changes in other Rho proteins, not measured here, due to compensatory adaptations, since Rho proteins tend to function as collective groups of signalling molecules as opposed to in the form of single gene transcripts. Furthermore, despite similarities between RhoA and RhoB sharing a high sequence homology and can activate many of the same signalling transduction systems, Ro61-8048 produced a highly selective change in RhoB, and RhoA remained unaffected. While the functions of RhoA and RhoB are closely related, they are often observed to produce opposing effects.

These opposing roles may be due to their post-translational modifications at the C-terminal, where they are prenylated to form farnesylated (FF) or geranylgeranylated (GG) isoforms of RhoB, while RhoA exclusively exists as RhoA-GG. These post-translational modifications enhance their interaction with specific membrane compartments (Ridley, 2006), often restricting RhoA to cytosolic membranes while RhoB is largely localised in early-endosomes and nuclear membranes. Therefore, this difference in post-translational modification and localisation may provide a mechanism by which RhoA and RhoB occasionally exert selective and differential roles.

#### *1.4.7 Poly(I:C) Induces no Changes in the Netrin Protein*

Netrins are a family of developmentally regulated proteins involved in axonal guidance of developing neurons. They are responsible for controlling cell and axon migration throughout the embryonic CNS (Cirulli and Yebra, 2007) by activating a variety of netrin receptors. In mammals, three netrins have been identified including: netrin-1, netrin-2 and netrin-3, in addition to glycosylphosphatidylinositol (GPI)-anchored membrane proteins, netrin-G1 and netrin-G2. Netrin ligands bind to receptors which include DCC (Deleted in Colorectal Cancer) family, the DSCAM (Down's Syndrome Cell Adhesion Molecule) family and the Unc5 homologue family, Unc5H1, Unc5H2, Unc5H3 and Unc5H4. Floor plate cells at the central midline of the embryonic neural tube secrete netrin-1 thereby forming a gradient of netrin proteins in the neuroepithelium. A growing axon either moves away from or towards a higher concentration of netrins (Rajasekharan and Kennedy, 2009), attracting some axon growth cones via activation of DCC receptors, while repelling others via activation of Unc5 receptors (Moore *et al.*, 2007). This bi-functional gradient guides growing axons to their ultimate target leading to the formation of synapses. DCC appears to be more important during development, while during puberty Unc5 homologues remain the predominant netrin receptors. DCC expression may be integral to dopamine function since highest levels are located in cell bodies and terminal regions of developing and adult dopaminergic neurons, suggesting a role for DCC in their organisation and function. Heterozygous mice expressing reduced levels of DCC, or its ligand netrin-1, exhibit profound abnormalities within mesocorticolimbic dopamine circuitry and function accompanied by behavioural abnormalities emerging after puberty (Manitt *et al.*, 2010; Xu *et al.*, 2010; Grant *et al.*, 2009). Therefore, given the close relationship between DCC and dopaminergic neurons, and that maternal infection substantially compromises the integrity of this system, we sought to examine whether poly(I:C) could induce changes in netrin receptors. However, protein expression for Unc5

homologues and DCC in embryos and P21 animals remained comparable with controls, implying deficits in dopaminergic pathways are not related to dis-regulated netrin receptors.

#### *1.4.8 Ro61-8048 Reduces Unc5H3 in the Cerebellum of P60 Animals*

Several reports of schizophrenic brains consistently and frequently observe substantial re-organisation of neuronal connections (Kannan *et al.*, 2009; Chen *et al.*, 2013), implying altered signalling of netrins and their receptors, since their primary developmental role exists in guiding axons to their target thereby forming normal functional connections. Consistently, altered expression of netrins is reported in the temporal lobe and hippocampus in schizophrenia and bipolar disorder (Eastwood *et al.*, 2008). Therefore, we sought to examine whether disruption of the kynurenine pathway could affect netrin receptors. Assessment of netrin proteins in embryo brains at 5 and 24 h showed no significant changes, while P21 animals similarly showed no change. However, consistent with Ro61-8048 inducing delayed changes, Unc5H3 was substantially reduced in the cerebellum of P60 animals. This demonstrates Ro61-8048 can induce significant impairments in netrin receptors during adulthood, suggestive of deficits in neuronal connectivity. Since the relative expression of netrin receptors determines the ultimate direction a developing axon will take during axonal guidance, the significant loss of Unc5H3 receptors imply reduced repelling rates of the axon growth cone thereby precipitating a ‘mis-wiring’ of the cerebellar cortex, which is consistent with similar reports of altered neuronal connectivity reported within schizophrenic brains, including cortical, hippocampal and cerebellar regions (Harrison, 2004; Kannan *et al.*, 2009; Chen *et al.*, 2013), contributing towards cognitive deficits.

#### *1.4.9 PolyI(C) Administration Induces no Change in TH Protein Expression*

Tyrosine hydroxylase is an enzyme continuously being utilised as an indication of dopaminergic activity, with increased levels expressed in schizophrenia. This is consistent with other groups reporting increases in DA and TH when treated with poly(I:C) (Meyer *et al.*, 2008c; Vuillermot *et al.*, 2010) and LPS (Romero *et al.*, 2008; Fan *et al.*, 2011b), while another independent group found an increase TH, DA and its metabolites including dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the nucleus accumbens (Aguilar-Valles *et al.*, 2010), suggesting the increase in TH is involved in the increased synthesis of DA and its turnover rate, as opposed to NA synthesis. This predisposed dopaminergic damage, producing compromised neurological responses, may also enhance adult susceptibility to develop neurodegenerative disorders, like PD, triggered by

environmental insults later in life, since LPS and poly(I:C) treatment in rodents enhances the vulnerability of nigrostriatal dopaminergic neurons to non-toxic doses of commonly used pesticides during adulthood (Fan *et al.*, 2011a; Bobyn *et al.*, 2012; Tien *et al.*, 2013). This may be mediated by increased oxidative metabolism of DA producing a highly reactive metabolite, a dopamine quinone, which induces cell death by covalently modifying parkin (LaVoi *et al.*, 2005), a ubiquitin E3 ligase enzyme, leading to the selective degeneration of dopaminergic cells in the substantia nigra (Petrucci *et al.*, 2002) by increasing oxidative stress and mitochondrial dysfunction (Lo Bianco *et al.*, 2004; Yasuda *et al.*, 2007; Rothfuss *et al.*, 2009), a mechanism thought to contribute to the early onset of Parkinson's disease (reviewed in von Coelln *et al.*, 2004). However, in contrast to previous studies, we failed to show an increase in protein expression of TH following prenatal treatment to poly(I:C) in embryo brains at 5 h or in P21 animals. The lack of change here may be related to examination of whole halves of the cerebral hemispheres, where subtle localised changes, in discrete brain regions, remain out-with the level of detection by western blotting.

#### *1.4.10 Prenatal Disruption of Kynurenines has no Effect on TH Expression*

Similarly when the protein expression of TH was examined following prenatal treatment with Ro61-8048, no changes were found in the embryos or at P21. The expression of TH is abundant in many regions of the brain including in most regions of the midbrain, cerebral cortex, basal ganglia, hippocampus, hypothalamus and cerebellum, therefore, the analysis was restricted to more regional areas in P60 animals. However, the protein content of TH remained comparable with controls when examined in the hippocampus, cortex, midbrain and cerebellum. This does not seem consistent with the hypothesis that maternal alterations of kynurenine levels may predispose the developing offspring to heightened risks of psychiatric disorders developing during adulthood, since prenatal disruption of kynurenines fail to precipitate one of the most basic deficits underlying schizophrenia, an increased activity in TH. While it may be argued should disruption of kynurenine metabolites during development be proposed as a neurodevelopmental model for schizophrenia, it should be efficacious in, and have a propensity towards, disrupting the dopaminergic transmitter system. Therefore, considering the current finding that the KMO inhibitor, Ro61-8048, failed to regulate the activity of TH, in addition with no change in the expression of GluN1 reported previously, this suggests that modulation of the kynurenine pathway during development may likely be involved in more subtle aspects of schizophrenia including cognition, neurogenesis and mild behavioural deficits, as opposed to modulating transmitter systems. This is supported since most changes observed with Ro61-8048 treatment are associated with neurodevelopmental proteins largely involved in neurogenesis, axonal guidance and synaptogenesis, aspects with a primary concern in cognition (Feldon and Folsom, 2009).

It must be considered however, that although no change was observed in TH, this does not necessarily mean no changes occurred in the dopamine transmitter system, since TH is also relevant to the synthesis of norepinephrine (NE) and may have little relevance to dopamine in our study, even though it is being utilised as a measure of dopaminergic activity. A limitation here is the present study failed to examine other dopamine related molecular targets including DA breakdown enzymes, metabolites or dopamine transporters to conclusively evaluate the role of DA following disruption of kynurenines. Furthermore, since considerable variations have been reported in the expression pattern of TH and DA even in subcellular locations of the striatum (Romero *et al.*, 2008; Meyer *et al.*, 2008c; Aguilar-Valles *et al.*, 2010; Vuillermot *et al.*, 2010) often in opposite directions, a measure of their immunoreactivity using immunocytochemistry as the principal technique may be more useful in localising these changes as opposed to examining the overall protein content

#### 1.4.11 Poly(I:C) Induces no Change in the Serotonin (5HT-2<sub>C</sub>) Receptors

Schizophrenia is recognised as a disorder involving alterations in many transmitter systems including dopamine, glutamate and serotonin. Since the initial dopamine hypothesis failed to account for all the symptoms of schizophrenia, the importance of the serotonin receptors became increasingly recognised when atypical second-generation anti-psychotics like clozapine, frequently considered the ‘gold standard’ amongst anti-psychotics, were associated with significant improvements in negative symptoms, while inducing negligible extrapyramidal side effects and tardive dyskinesia (Miyamoto, 2005; Newman-Tancredi and Kleven, 2010), were found to antagonise serotonin receptors (Meltzer and Massey, 2011). It is now widely accepted that a variety of serotonin receptors are involved in schizophrenia, including 5HT-1 and 5HT-2 receptors. While 5HT-1<sub>A</sub> receptors have received most attention in their relationship with schizophrenia, the serotonin 5HT-2<sub>C</sub> receptor is responsible for producing hallucinations. *In vivo* models of schizophrenia induced by PCP show significant improvements in response to the 5HT-2<sub>C</sub> receptor agonist, WAY-163909 (Dunlop *et al.*, 2006), while inverse agonists restore novel object recognition in PCP-treated rats (Meltzer *et al.*, 2011). Models of maternal immune activation, capable of precipitating schizophrenia-like symptoms *in vivo*, have demonstrated a range of behavioural abnormalities including neurochemical alterations of the serotonergic system. Prenatal LPS exposure reduces cortical mRNA levels of 5HT-1<sub>A</sub> and 5HT-1<sub>B</sub> receptors in neonatal pups at P3 (Baharooni *et al.*, 2010), while a similar report of prenatal stress showed schizophrenia-like alterations in 5HT-2<sub>A</sub> (Holloway *et al.*, 2013). We therefore sought to examine if prenatal exposure with poly(I:C)



could induce any changes in 5HT-2<sub>C</sub> receptors that may be involved in mediating schizophrenia-like behavioural abnormalities in the offspring. When its protein expression was examined in the embryos at 5 h following treatment and in P21 animals, the levels of 5HT-2<sub>C</sub> remained comparable with control animals. Since LPS down-regulated 5HT-1<sub>A</sub> and 5HT-1<sub>B</sub> mRNA transcripts in neonatal animals at much earlier time point of P3 (Baharooni *et al.*, 2010), it remains plausible that maternal immune activation with poly(I:C) may induce similar changes in 5HT-2<sub>C</sub> receptors during early postnatal development as opposed to later stages. Furthermore, changes in the serotonin system appear to have a complex set of interactions where differential changes are reported in highly localised regions, and therefore may be undetectable with the simple examination of entire halves of the cerebral cortices.

Although the results from the present study imply 5HT-2<sub>C</sub> is not responsible for mediating deficits in behaviour following maternal immune activation with poly(I:C), this does not categorically exclude it from being involved since maternal exposure to poly(I:C) during gestation day 9, decreased 5-HT in the nucleus accumbens (NAc) by 38%, lateral global pallidus (LGP) by 30% and in the hippocampus by 42% in the adult offspring, including reductions in 5-hydroxyindoleacetic acid (5-HIAA), a major metabolite of 5-HT breakdown, in the lateral global pallidus and hippocampus (Winter *et al.*, 2009). Also, in animal models of immunologically-induced fatigue, poly(I:C)-treated rats show increases in mRNA of serotonin transporters (5-HTT) on days 1 and 8 following treatment, while extracellular concentrations of 5-HT decrease in the medial prefrontal cortex (Katafuchi *et al.*, 2005), presumably accounted for by the increase in 5-HTT. It is likely the kynurenine pathway may be a major contributor towards the loss of 5-HT observed in these studies, since activation of the maternal immune response induces activation of indoleamine-2,3 dioxygenase (IDO) to initiate the metabolism of tryptophan, an essential amino acid required for 5-HT synthesis. Studies have consistently shown an increase in IDO activity during infectious conditions depletes tryptophan levels as a protective mechanism. However, this negatively impacts the serotonin system by inducing a loss in central 5-HT, a proposed mechanism increasingly becoming recognised as mediating the link between immune activation and the subsequent depressive-like symptoms occurring in humans and rodents.

Depressed patients display a correlation for an increase in pro-inflammatory cytokines, like IL-6 and c-reactive protein, with the symptoms of major depression (Dobos *et al.*, 2012), while inflammation within the periphery induced by LPS, in human volunteers and rodent models, precipitates profound changes in cognitive function, mood and behaviour relevant to depression (Reichenberg *et al.*, 2001; Krabbe *et al.*, 2005; O'Connor *et al.*, 2009). Furthermore,

increased IDO enzyme activity and kynurenine concentrations have been correlated with inflammation-associated depression (Dantzer *et al.*, 2011; Christmas *et al.*, 2011; Myint, 2012; Maes *et al.*, 2012). This is further supported by studies demonstrating the genetic deletion of IDO abrogates inflammation-dependent behavioural changes that model depression in models of acute inflammation with LPS, while these observations have been extended to models of chronic inflammation induced by peripheral infection (O'Connor *et al.*, 2009; Salazar *et al.*, 2012). Intracerebroventricular injection of TNF- $\alpha$  and LPS induces depressive-like behaviours in rodents associated with the induction of cytokines and IDO expression (Kaster *et al.*, 2012; Dobos *et al.*, 2012). This has been confirmed recently where a single LPS intracerebroventricular injection in mice increase central kynurenine and IDO activation associated with the subsequent development of depressive-like behaviours. The role of IDO in mediating this response was confirmed in IDO knockout mice or wild-type mice pre-treated with the IDO inhibitor 1-methyl-tryptophan (1MT), as mice failed to develop depressive-like symptoms when compared with control animals administered LPS alone (Lawson *et al.*, 2013).

Collectively, these studies illustrate that poly(I:C) and LPS, whether exposed prenatally or in neonatal rats, is efficacious in inducing a reduction in the neurotransmitter 5-HT in many cortical regions, via activation of IDO and an increase in central kynurenines, thereby accounting for the onset of depressive-like behaviours. The significance here is that although no change in 5HT-2<sub>C</sub> was found in our experiments following prenatal exposure to poly(I:C), the reduced availability of 5-HT may equally be as significant and physiologically relevant as a decrease in the receptor, since a decrease in 5-HT would ultimately lead to reduced activation of its receptors, including the 5HT-2<sub>C</sub> receptor. Reduced activation of 5HT-2<sub>C</sub> receptors may underlie the increased susceptibility of neuropsychiatric disorders including major depression and schizophrenia, since both disorders principally rely upon the reduced activity of 5HT-2<sub>C</sub> (Dunlop *et al.*, 2006; Miyamoto, *et al.*, 2005; Newman and Kleven, 2010). Furthermore, behavioural deficits in prepulse inhibition (PPI) during schizophrenia, and following maternal immune activation with poly(I:C) or LPS, may be due to the loss of central 5-HT, since serotonin in the nucleus accumbens plays an essential role in the regulation and modification of PPI (Swerdlow *et al.*, 2001), while central 5-HT depletion is known to disrupt PPI in rats (Prinssen *et al.*, 2002).



#### 1.4.12 Ro61-8048 has no Effect on Serotonin (5HT-2<sub>c</sub>) Receptor Protein Expression

5HT-2<sub>c</sub> receptors may be of considerable interest in relation to schizophrenia, since their primary location appears to be the choroid plexus, striatum and hippocampus (Clemett *et al.*, 2000; López-Gimenez *et al.*, 2002), where *in vivo* studies show 5HT-2<sub>c</sub> is an important regulator of LTP and behaviour (Tecott *et al.*, 1998). Since such abnormalities are likely to contribute towards subtle deficits in cognitive abilities, and the developmental effects of Ro61-8048 have not been documented, we sought to examine whether modification of the kynurenine pathway during development may induce changes in serotonin systems. However, no changes were detected in embryo brains at 5 and 24 h. When examined in P21 animals including in the hippocampus, cortex, midbrain and cerebellum of P60 animals, 5HT-2<sub>c</sub> levels remained comparable with controls. It is therefore unlikely 5HT-2<sub>c</sub> receptors are involved in contributing towards deficits in brain and behaviour following disruption of the kynurenine pathway, and further supports the theory that developmental changes in kynurenines may be involved in subtle aspects of schizophrenia including cognition and plasticity, as opposed to more overt aspects of hallucinations.

#### 1.4.13 $\alpha$ -Synuclein ( $\alpha$ -Syn) is Substantially Reduced by poly(I:C)

$\alpha$ -Synuclein is heavily implicated in the formation of lewy bodies observed in neurodegenerative disorders including Parkinson's and Alzheimer's disease, where  $\alpha$ -synuclein monomers abnormally processed along the ubiquitin-proteasome pathway are rendered insoluble forming oligomer aggregates. Synucleinopathies are toxic to dopaminergic neurons and can induce neuronal loss either directly or indirectly via oxidative stress and mitochondrial dysfunction (Chinta *et al.*, 2010). Lewy body pathology is associated with deficits in synaptic proteins including reductions in syntaxin and SNAP-25 in the neocortex, while SNAP-25 and  $\alpha$ -synuclein are reduced in the medial temporal lobes (Mukaetova-Ladinska *et al.*, 2009). Large oligomeric or protofibril forms of  $\alpha$ -synuclein inhibit neuronal SNARE-mediated vesicle lipid mixing by preferentially binding to the N-terminal domain of synaptobrevin-2 (VAMP-2) thereby blocking the formation of the SNARE complex (Choi *et al.*, 2012). Therefore, since synucleopathies can induce deficits in the SNARE complex, this may be a mechanism that may contribute towards a reduced dopamine (DA) and acetylcholine (ACh) release observed in Parkinson's disease. Interestingly, another study suggested SNARE dysfunction itself may be the initial trigger of mislocalisation and accumulation of  $\alpha$ -synuclein (Nakata *et al.*, 2012). However, since the involvement of  $\alpha$ -synuclein in these neurodegenerative disorders appear largely due to its increase, while our experiments detected a substantial loss,  $\alpha$ -synuclein is not involved in such processes.

However, this may be an overly simplistic analysis of the data as to what is considered an increase or decrease in real physiological terms. For example,  $\alpha$ -synuclein forms a pool of soluble proteins representing the total pool of physiologically active proteins contributing towards physiological functions, while  $\alpha$ -synuclein that becomes insoluble and a component of lewy bodies becomes physiologically inactive and is removed from the active pool. Therefore this rise in 'dead' insoluble  $\alpha$ -synuclein detected in lewy bodies will subsequently lead to the overall decline in the physiological active soluble forms of  $\alpha$ -synuclein, which is consistent with our result. This would largely depend upon whether the antibody used recognises mutant forms of insoluble  $\alpha$ -synuclein or if it is limited to detecting the physiologically active soluble form. However, in spite of the antibody specificity, which is speculative in the context of our experiments, Parkinson's disease can be considered in terms of both an increase and a decrease in  $\alpha$ -synuclein relating to their relative forms in which they exist. This is consistent with the majority of reports detecting an increase in  $\alpha$ -synuclein in Parkinson's disease localised within lewy bodies, thereby accounting for an increase of inactive  $\alpha$ -synuclein, while a 50% reduction in mRNA expression of  $\alpha$ -synuclein at the cellular level of neurons in the substantia nigra and of the frontal cortex in Parkinson's disease (Kingsbury *et al.*, 2004) may be accounted for by the physiologically active pool of soluble  $\alpha$ -synuclein remaining. Both of these mechanisms can contribute to the pathophysiology of Parkinson's disease and not only due to the presence of lewy bodies. Indeed, a decline in striatal dopamine release may be in part due to the selective neurodegeneration exerted on dopaminergic neurons, as induced by lewy body synucleopathy, but may also be due to the reduced pool of soluble presynaptic  $\alpha$ -synuclein. Particularly relevant to this, are studies in transgenic mice lacking the  $\alpha$ -synuclein gene, which show changes in activity-dependent modulation of neurotransmitter release (Abeliovich *et al.*, 2000) and a reduced neurotransmitter release in response to repeated stimulation (Cabin *et al.*, 2002), thereby linking soluble presynaptic  $\alpha$ -synuclein with synaptic transmission (Gitler and Shorter, 2007). These studies suggest a reduced expression of  $\alpha$ -synuclein may contribute to deficits in transmitter release. Furthermore, since one study reported a 50% reduction in mRNA expression of  $\alpha$ -synuclein in neurons of the substantia nigra and frontal cortex in Parkinson's disease (Kingsbury *et al.*, 2004), this may function to reduce dopamine and acetylcholine neurotransmission within these regions, thereby contributing towards the cognitive decline associated with Parkinson's disease.

Despite substantial links with  $\alpha$ -synuclein in the pathology of Parkinson's and Alzheimer's disease, emerging studies are increasing linking  $\alpha$ -synuclein as a biomarker of

schizophrenia and bipolar disorder. This may not be surprising, since  $\alpha$ -synuclein is a synaptic protein and many synaptic proteins are dis-regulated during these disorders including VAMP-1 (synaptobrevin), synaptophysin, syntaxin and SNAP-25. Although the precise function of  $\alpha$ -synuclein is unknown, biochemical studies show a reduction in  $\alpha$ -synuclein in Broadman's area 9 in both chronic schizophrenia and bipolar disorder (Bernstein *et al.*, 1998). Similarly, another independent group found  $\alpha$ -synuclein was significantly reduced in post-mortem brain tissue of patients with bipolar disorder (Gray *et al.*, 2010), while another group showed down-regulation in mRNA levels of  $\alpha$ -synuclein in lymphocytes of schizophrenia patients (Noori-Daloi *et al.*, 2010). These results are entirely consistent with our finding of a reduced expression of  $\alpha$ -synuclein in a developmental model of schizophrenia. On the contrary, one proteomic analysis reported a significant increase in  $\alpha$ -synuclein in layer 2 of the insular cortex in schizophrenia (Pennington *et al.*, 2008b), suggesting changes are likely to be region specific and may correspond to disease progression. While these alterations may perturb synaptic function, the precise role of  $\alpha$ -synuclein during these disorders remains unclear. Since  $\alpha$ -synuclein can affect dopamine homeostasis, and maternal immune activation induces changes within the dopaminergic system, the poly(I:C)-induced down-regulation of  $\alpha$ -synuclein during development may be involved in the loss of this homeostatic control over dopaminergic neurons, and may account for another mechanism by which prenatal infection induces significant alterations within the dopaminergic system.

#### 1.4.14 *Changes in Kynurenines do not Regulate the Activity of $\alpha$ -Synuclein*

Due to the significant involvement of the kynurenine pathway in Parkinson's disease, and the finding that infections and pro-inflammatory cytokines can induce significant nigrostriatal degeneration (Chakrabarty *et al.*, 2011), the protein expression of  $\alpha$ -synuclein was examined following prenatal treatment with Ro61-8048. Interestingly, although poly(I:C) substantially reduced its protein expression, Ro61-8048 produced no effect in the embryos when examined at 5 and 24 h. Since Ro61-8048 usually produces delayed alterations during later stages of development, its expression was examined in P21 and P60 animals. Similarly, in P21 animals the expression of  $\alpha$ -synuclein remained comparable with control animals, implying that the reduction seen in poly(I:C) at P21 is not mediated by the kynurenine pathway. Furthermore, regional experiments in the hippocampus, cortex, midbrain and cerebellum of P60 animals also produced no effect on the expression profile of  $\alpha$ -synuclein. This demonstrates the role of the kynurenine pathway in Parkinson's disease does not regulate  $\alpha$ -synuclein activity, and since this is a downstream target comprising an end product producing synucleopathies, kynurenine involvement may regulate neuronal activity and neurodegeneration, as opposed to the formation of large protofibril deposits. Therefore, the

absence of a change here is in conformity with the hypothesis that kynurenines may be at the forefront of cognitive dysfunction thereby regulating neuronal activity.

#### *1.4.15 Poly(I:C) does not Affect DCX Protein Expression*

Doublecortin is a microtubule-associated protein commonly used as a marker of neurogenesis due to its primary location in newly formed neurons (Couillard-Despres *et al.*, 2005). DCX is required for the normal migration of differentiating neurones in the cerebral cortex, and since this process declines with age, due to much lower levels of neurogenesis and neuronal migration, the expression of DCX also declines, detecting low levels of this protein in the adult brain. Deficits in neurogenesis and neuronal migration during development is associated with significant abnormalities in cortical development (Reiner *et al.*, 1993; Gleeson *et al.*, 1998; Francis *et al.*, 1999), and disruption of DCX protein expression may contribute to a variety of diseases including mental retardation (Gleeson and Walsh, 1997; Berg *et al.*, 1998; Gleeson *et al.*, 1999). Since reduced levels of neurogenesis occurs in schizophrenia, possibly accounting for reduced cortical and hippocampal volume, we sought to examine the level of neurogenesis following treatment with poly(I:C), to find DCX protein expression remained unchanged in embryo brains and P21 animals. This is in contrast to other groups reporting reduced levels of hippocampal neurogenesis in postnatal animals following prenatal or neonatal treatment to LPS and poly(I:C) (Ekdahl *et al.*, 2003; Cui *et al.*, 2009; Graciarena *et al.*, 2010; Wolf *et al.*, 2011). The lack of change of DCX in our experiments, in contrast to other groups, may be attributed to differences in the strain of animals used in these studies, or differences in timing and the level of severity of infection and dosage of immunogenic stimulants administered.

#### *1.4.16 Ro61-8048 Administered Prenatally Affects Adult Neurogenesis*

When the protein expression of DCX was examined in Ro61-8048-treated animals, no changes were seen in the embryo brains at 5 and 24 h, suggesting Ro61-8048 does not induce deficits in neurogenesis or neuronal migration during embryogenesis. However, while no changes were seen in the protein expression of DCX at P21, it was significantly increased in the hippocampus of P60 animals only. Although post-mortem studies indicate a reduction in the number of dividing cells in the subventricular zone of the dentate gyrus of individuals with schizophrenia, suggesting that deficits in neurogenesis in the adult hippocampus may be involved in the disorder, in contrast to these reports, the increase in DCX in the P60 hippocampus implies the increased rate in new neurogenesis within Ro61-8048-treated offspring. However, a parallel study in the laboratory indicated no change in the immunoreactivity of doublecortin-positive cells in the dentate gyrus of the hippocampus, with

a decrease in the number of neurons bearing complex dendrites, suggesting that the overall levels of neurogenesis within the dentate gyrus remained comparable with control animals. Therefore, the increase in expression of DCX in the P60 hippocampus may reflect an increase of recently generated neurons migrating to other regions of the hippocampus. This is supported by an increase in Neu-N staining observed in the CA1 region, implying an increase in the total neuronal number, thereby potentially accounting for the increased protein expression.

This increase may be related to electrophysiological changes observed in the CA1 hippocampus of these animals, measured alongside in a parallel study. While no overall changes in long-term depression (LTD) were found, the delayed recovery from low frequency stimulation may be related to the link between doublecortin and GABAergic function. Indeed, mutations or loss of doublecortin to produce neuronal hyperexcitability is caused by the reduction of synaptic inhibition (Kerjan *et al.*, 2009). Since doublecortin is co-localised with GABA, its synthetic enzyme glutamate decarboxylase, or parvalbumin, a marker for GABA-releasing neurons (Cai *et al.*, 2009), this lends support to the proposal that neurons expressing doublecortin may be primarily destined to differentiate into GABAergic interneurons (Wu *et al.*, 2008; Xiong *et al.*, 2008), while doublecortin may also mediate the early migration of inhibitory interneurons in the cortex (Kappeler *et al.*, 2006; Friocourt *et al.*, 2007). Therefore, the increase in doublecortin observed in the hippocampus at P60 may represent an increase in the number of GABAergic interneurons in the CA1 region thereby affecting neuronal hyperexcitability by reducing synaptic inhibition. This is also consistent with reports suggesting increased hyperexcitability in the CA1 hippocampus may contribute towards its volume loss and shrinkage, while being involved in core symptoms of schizophrenia including hallucinations and delusions (Zierhut *et al.*, 2012). Furthermore, since newly generated neurons are linked to the functioning of the hippocampus, critically involved in certain aspects of cognition, including learning and memory, these processes may also be adversely affected.

#### *1.4.17 Poly(I:C) has no Effect on the Expression of Synaptic Proteins*

Normal synaptic function is imperative for the normal development of the brain and is dependent upon a wide array of protein interactions forming a network which together function collectively to mediate the transport, fusion and recycling of synaptic vesicles. These protein networks bring together vesicular and presynaptic membranes within close proximity to drive fusion pore formation and expansion facilitating vesicular fusion and release of their luminal content (Lynch *et al.*, 2008). This process occurs in the release of transmitters from

nerve terminals, while the same protein complexes are increasingly becoming recognised for their roles during endocytosis, to recover and recycle synaptic vesicles (Schwartz, 2004). The aetiology of psychiatric disorders including schizophrenia and bipolar disorder are believed to involve the aberrant regulation of synaptic function, since many components of the SNARE complex and other proteins relating to this complex are significantly altered in post-mortem brains, including VAMP-1 and synaptophysin (Eastwood and Harrison, 2001; Vawter *et al.*, 2002; Halim *et al.*, 2003; Beasley *et al.*, 2005; Scarr *et al.*, 2006). However, in contrast, our experiments failed to detect any change in the protein expression of synaptic proteins in the embryos or at P21. It should be noted that the lack of or exaggerated changes reported for VAMP-1 in post-mortem tissue may be related to the fact that protein content degrades rapidly following death. Furthermore, confounding factors apply to most post-mortem studies of bipolar disorder, since patients received anti-psychotic, lithium or anti-depressant drug treatment, while the latter treatment affects synaptic proteins including the expression of VAMP-2, which may induce compensatory adaptations in VAMP-1.

Although our experiments detected no change in synaptophysin in the embryos or P21 animals following poly(I:C), this result is consistent with a similar study of prenatal stress, where maternal restraint or corticosterone injections reduced synaptophysin expression in P7 and P14 animals only (Afadlal *et al.*, 2010), and not at later stages. Other models of restraint stress have similarly reported reductions in VAMP-1 or synaptophysin in the hippocampus of rodents (Thome *et al.*, 2001; Xu *et al.*, 2004; Muller *et al.*, 2011). Furthermore, since most studies identified changes in discrete regions of the brains, it is likely that discrete changes are masked in our experiments since the entire half of the cerebral hemisphere was examined. Therefore, even if there was a decrease in the hippocampus, as seen following prenatal stress, and an increase in another region, overall this would show no net change in protein expression in our experiments. This may account for why no change was seen in synaptophysin despite a reduction in GluN1, since the pharmacological blockade of the NMDA receptor with MK-801 during P7 is associated with reduced synaptophysin mRNA levels in the thalamus during adulthood (Harrison *et al.*, 2003).

#### *1.4.18 Ro61-8048 Induces no Change in the Expression of Synaptic Proteins*

Synaptic dysfunction is a prominent pathology in schizophrenia, and the pharmacological blockage of NMDA receptors during development produce a substantial synaptic loss; therefore, since Ro61-8048 treatment significantly increases central levels of KYNA capable of antagonising the NMDA receptor, the subsequent effect upon synaptic development was assessed by examining changes in protein expression. However, no changes



were seen in the embryos or postnatal animals of synaptic proteins suggesting synapse machinery and transmitter release remains unaffected. This is consistent with the lack of change in VAMP-1 reported in post-mortem brains in the anterior frontal cortex (Honer *et al.*, 2002), the frontal, temporal and parietal cortices (Gabriel *et al.*, 1997), the cerebellum (Mukaetova-ladinska *et al.*, 2002) and Broadman's area 9 in schizophrenia patients (Scarr *et al.* 2006). However, since one post-mortem study reported a loss of VAMP-1 in schizophrenia patients (Halim *et al.*, 2003), this suggests alterations in VAMP-1 are not a global change but may only be relevant in distinct regions of the brain critically depending upon the relative stage of the disease. Clinical studies may also provide support for the general loss of VAMP-1 during schizophrenia, since anti-psychotic drug treatment regulates synaptic protein expression (Barr *et al.*, 2006). Clozapine, an atypical anti-psychotic, increases VAMP-1 in human brain aggregates, rodent models (Chana *et al.*, 2009), and the rat striatum (Barakauskas *et al.*, 2010). Therefore, it is possible anti-psychotic treatment during schizophrenia may also serve to restore neuronal and synaptic function by correcting deficits in synaptic proteins that are disrupted.

#### *1.4.19 Ro61-8048 Increases VGLUT Terminals While VGAT Remains Unaffected*

Since several reports show altered signalling pathways in the hippocampus including a range of neurochemical alterations in GABA and glutamate systems in schizophrenia, the function of excitatory and inhibitory neuronal function was examined in the hippocampus of P60 animals. Synaptic release of glutamate is the major pathway of excitatory neurotransmission in the mammalian brain, where glutamatergic neurons express the vesicular glutamate transporters VGLUT-1, VGLUT-2 and VGLUT-3 within axon terminals. The expression of vesicular transporters for glutamate are primarily responsible for the uptake of glutamate into synaptic vesicles, a process driven by a proton electrochemical gradient generated by the vascular  $H^+$ -ATPase (Kaneko *et al.*, 2002). The presence of transporters within nerve terminals of neurons define the glutamatergic phenotype since forced expression of VGLUT-1 and VGLUT-2 in inhibitory neurons induces quantal glutamate release (Takamori *et al.*, 2001). Despite close similarities between excitatory glutamate transporters in substrate specificity, kinetics and pharmacology, they differ in their expression profiles. VGLUT-1 and VGLUT-2 are predominantly expressed in the adult brain, displaying a roughly complementary expression profile where they exist on separate neurons or co-expressed on the same neuronal populations. This is supported by VGLUT-1 knockout mice showing continued glutamate release due to the presence of VGLUT-2 in the hippocampus. While the expression of VGLUT-1 is more important in the cerebral and cerebellar cortices including

the hippocampus (Balschun *et al.*, 2010), VGLUT-2 is mainly located in the diencephalon, brain stem, thalamus and midbrain (Moechars 2006; Kubota 2007). Interestingly, a developmental switch between the expression of VGLUT-1 and VGLUT-2 occurs within the hippocampus, cortex and cerebellum. While VGLUT-2 is especially important during embryonic and neonatal development, it is largely replaced by the expression of VGLUT-1 during adulthood (Fremeau *et al.*, 2004). Due to the regulatory roles of these transporters on transmission, VGLUT-1 and VGLUT-2 have been widely adopted as indicators of excitatory glutamatergic transmission, where the relative densities of these transporters are indicators of quantal size and transmitter release.

Interestingly, the relative density of the excitatory vesicular transporters VGLUT-1 and VGLUT-2 were substantially increased in the CA1 hippocampus at P60, demonstrating Ro61-8048 treatment during development significantly alters excitatory glutamatergic transmission during adulthood. The relative density of VGLUT-3 was not since this isoform is less widely expressed, and often located in cholinergic, serotonergic and GABAergic neurons, suggesting involvement in unconventional glutamate transmission. The significant increase in VGLUT-1 and VGLUT-2 in the CA1 hippocampus could be predictive of an increase in the quantal size of glutamate release increasing glutamatergic transmission. This is supported by VGLUT-1 knockout mice showing reduced excitatory postsynaptic potentials, with reduced glutamate release and synaptic transmission in the stratum radiatum, presumably resulting from the loss of glutamate uptake (Fremeau *et al.*, 2004). This is consistent with another study where the targeted deletion of VGLUT-1 drastically reduces glutamate transmission, with a specific reduction in quantal size (Wojcik *et al.*, 2004), an effect rescued by overexpression of VGLUT-1. Therefore, since excitatory glutamate transporters determine the amount of glutamate loaded into vesicles and released, the highly significant increase of VGLUT-1 and VGLUT-2 in the P60 hippocampus may be predictive of an enhanced excitatory glutamatergic transmitter release.

Although the initial hypothesis proposed that alterations in kynurenines during development may enhance the risk of schizophrenia during adulthood, the increase in glutamate transporters, suggestive of an increase in glutamate activity, is not consistent with findings of a reduced excitatory activity, especially within the hippocampus during schizophrenia (Harrison *et al.*, 2006). In contrast, mRNA levels of VGLUT-1 are reduced in the hippocampus in schizophrenia (Eastwood and Harrison, 2004), an effect becoming more pronounced with age progression. Similarly, a reduced VGLUT-1 expression is reported in the caudate nucleus (Nudmamud-Thanoi *et al.*, 2007) and anterior cingulate cortex in



schizophrenia, while VGLUT-2 remains unaffected (Oni-Orisan *et al.*, 2008). However, since anti-psychotics regulate VGLUT activity (Nudmamud-Thanoi, *et al.*, 2007), the loss of VGLUT terminals in schizophrenia may be related to the use of anti-psychotic treatment, since haloperidol treatment in rats significantly reduces VGLUT-1 mRNA expression in the temporal cortex (Uezato *et al.*, 2009). This suggests the therapeutic efficacy of anti-psychotics in treating schizophrenia may also function to restore the balance of excitatory glutamate transporters, implying schizophrenia may indeed be associated with an increase in VGLUT transporters, which is consistent with our findings. This may potentially explain the conflicting reports of why reductions and increases are reported in schizophrenia for VGLUT transporters (Talbot *et al.*, 2004; Eastwood and Harrison, 2005; Fung *et al.*, 2011b). Furthermore, the effects of anti-psychotics on reducing VGLUT terminals is also consistent with the finding that VGLUT reduction is enhanced with age and disease progression (Eastwood and Harrison, 2004; Harrison *et al.*, 2006), which is consistent with the long-term use of anti-psychotics. It remains possible that during schizophrenia, VGLUT transporters may function to reduce overall glutamatergic activity, since uptake of glutamate into nerve terminals from the synaptic cleft, following glutamate release, is a mechanism terminating the excitatory actions of glutamate. The increase in VGLUT terminals in schizophrenia may reflect the enhancement of glutamate activity termination thereby contributing towards a reduced overall excitatory function in the hippocampus, a theory more consistent with schizophrenia. Therefore, since prenatal modification of the kynurenine pathway induces substantial increases in the expression of VGLUT-1 and VGLUT-2 terminals, this further supports a developmental role for the kynurenine pathway in schizophrenia. While no attempt was made in the present study to determine the individual isoform of VGLUT that may be elevated following Ro61-8048, since VGLUT-1 is more prevalent in the hippocampus, the increase in glutamate transporters may relate towards the VGLUT-1 isoform, as opposed to VGLUT-2. Although the anatomical origin of VGLUT-positive terminals were not assessed in this study either, since different groups of afferent neurons project onto different regions of the dendritic surface (Ferrante *et al.*, 2013; Gidon & Segev, 2012), it is possible the increase in VGLUT terminals may be confined to a limited number of afferent sources.

The increase in the number of VGLUT terminals may also contribute to the findings in a parallel study, of a reduced paired-pulse inhibition in the CA1 region of the hippocampus of P21 animals following Ro61-8048 treatment (Forrest *et al.*, 2013). Although paired-pulse inhibition and facilitation are largely determined presynaptically (Rosenmund and Stevens, 1996; Zucker *et al.*, 1991), the final level of paired-pulse interaction is partly influenced by the number of excitatory and inhibitory terminals on the neurons being recorded. Therefore, the

substantial increase in excitatory VGLUT terminals may account for the reduced paired-pulse inhibition.

In order to confirm the increase in VGLUT terminals were not due to increases in synaptic contacts, VAMP-1 staining was assessed. Interestingly, since no change occurred in VAMP-1, this confirmed that the increase in VGLUT transporters represented an increase in their relative numbers while there is no change in the total number of synaptic contacts. The lack of change in VAMP-1 reported here is also consistent with no change seen in the protein expression of VAMP-1 in the P60 hippocampus. When co-localisation was assessed, there was no change in the co-localisation of VAMP-1 with VGLUT terminals or VAMP-1 with VGAT. Furthermore, the increase in VGLUT-1 and VGLUT-2 staining occurred in the absence of any change in the GABA vesicular transporters VGAT. Since VGAT is located on nerve terminals of GABA and glycinergic neurons responsible for the uptake of GABA (Chaudhry *et al.*, 1998; Minelli *et al.*, 2003; Bragina *et al.*, 2010), the absence of a change here not only suggests that changes in VGLUT terminals are a highly specific phenomenon, but also confirms the absence of a global gain or loss of synapses. This may therefore be a consequence of an increased ratio of dendritic branches available for synaptic contact since a parallel study showed primary and secondary dendrite lengths were significantly increased. The lack of change in VGAT suggests GABAergic signalling in the hippocampus remains unaffected, a finding conflicting with the reduced densities of GABA uptake sites and GABAergic parvalbumin-immunoreactive neurons in the hippocampus in schizophrenia (Reynolds *et al.*, 1990; Zhang and Reynolds, 2002), suggesting that Ro61-8048 cannot recreate all the deficits in hippocampal transmission relevant to schizophrenia. However, it is possible that while GABA transporters remained the same, there may be impairments in the levels of GABA synthesis, receptors or the rate of metabolism. Since this was not addressed in the present study, the current result assumes no alterations in GABAergic signalling following prenatal modification of the kynurenine pathway.

#### *1.4.20 Ro61-8048 Reduces Hippocampal Spine Density*

Dendritic spines are tiny protrusions emerging from the dendrites of most neurons in the brain. Classical dendritic spines consist of a head and a postsynaptic density connected to the dendritic shaft by a narrow neck, representing the main input for neurons behaving as subcellular compartments locally controlling signalling mechanisms, and involved in receiving and processing synaptic information (Fiala *et al.*, 2002). Spine density relates to the amount of connectivity between neurons with dendritic spines and the axons emerging from other

neurons thereby forming synaptic contacts. Dendritic spines are critically involved in establishing and maintaining these connections for neurons to accurately receive excitatory input, and are imperative for the normal functioning of neurons. Furthermore since dendritic spines compartmentalise calcium and other signalling components, they are known to be involved in synaptic efficacy (Nimchinsky *et al.*, 2002).

Hippocampal spines vary considerably in size and shape along dendritic segments, however, most spines have constricted necks with either larger mushroom heads or thin shaped with smaller heads (Harris *et al.*, 1992). Other spines include stubby protrusions emerging from the dendritic segment maintaining a head width equal to that of the neck width and length. Mushroom spines exhibit larger more complex postsynaptic densities with a higher density for glutamate receptors (Nicholson *et al.*, 2006), suggesting these spines are functionally stronger in their response to glutamate and local regulation of intracellular calcium, while smaller spines may be more flexible, enlarging or shrinking in response to subsequent activation (Bourne and Harris, 2007).

Since Ro61-8048 produced a variety of changes in the expression levels of proteins with known functions in neuronal development, we sought to examine whether these changes in protein expression, as induced by Ro61-8048, were also accompanied by alterations in spine density. Interestingly, when dendritic spines of pyramidal cells were examined in the P60 hippocampus, a significant reduction was seen in apical and basal dendrites, demonstrating the developmental regulation of kynurenines substantially reduces hippocampal spines, and may potentially reduce cognitive abilities during adulthood. Since NMDA receptors are intimately involved in spine and synapse formation, and knocking down NMDA receptors through RNA interference (RNAi) results in increased spine mortality and eventually elimination (Alvarez *et al.*, 2007), it may be predicted that elevation of KYNA during development, induced by Ro61-8048, may directly account for the loss of spines seen here. However, since blocking NMDA receptor signalling during development does not affect the emergence or density of spines (Alvarez *et al.*, 2007), changes in NMDA activity as a consequence to the increased concentrations of KYNA may have induced the loss of spines via changes in protein expression or the altered function of the NMDA receptor. In support of this is that spine density is affected by the GluN2B subunit of the NMDA receptor (Brigman *et al.*, 2010), which was increased in the P21 animals (Forrest *et al.*, 2013), and this could affect the relative densities of mushroom and thin spines on apical and basal dendrites. Furthermore, the metabotropic glutamate receptor mGluR5 also regulates spine density, while its loss is associated with an increase in spine density in the neocortex (Chen *et al.*, 2012). However,

since mGluR5 receptors affect the developmental switch from GluN2B-containing NMDA receptors to those expressing GluN2A subunits, this suggests that the NMDA may be the final convergent pathway in mediating the effects on spine density. Furthermore, the loss of RhoB in P21 animals may also contribute towards the loss of spines, as well as the increase in DISC-1 in the P60 cortex since they exert regulatory roles over spine formation, development and morphology (Ramsey *et al.*, 2011).

Hippocampal spines show structural plasticity as the basis for physiological changes in synaptic efficacy that underlie learning and memory. Long-term potentiation (LTP) alters spine number, shape and the subcellular composition of dendritic spines in the immature (Nagerl *et al.*, 2007) and mature hippocampus (Stewart *et al.*, 2005), while the converse phenomenon of long-term depression (LTD) decreases spine number and size (Zhou *et al.*, 2004). Spine plasticity in the hippocampus involves changes in the size and composition of the postsynaptic density, actin filaments and activity of glutamate receptors and ion channels. Dendritic spines play a prominent role in neuronal plasticity where several forms of learning are associated with increased numbers of dendritic spines (Leuner and Shors, 2004). Spatial training of adult rats increases learning ability associated with increased spine densities of pyramidal neurons in the CA1 region of the hippocampus (Moser *et al.*, 1994). Environmental enrichment during development also results in increased spine and synapse density on pyramidal cells in the occipital cortex and CA1 region of the hippocampus (Berman *et al.*, 1996). Since learning is associated with enhanced spine densities, the converse phenomenon remains true; showing the loss of spine density is associated with cognitive decline. While learning, memory and cognition are impaired with age progression, reduced spine densities in CA1 pyramidal neurons also occurs (von Bohlen und Halbach *et al.*, 2006; von Bohlen und Halbach *et al.*, 2010). Similarly, layer I of the prefrontal cortex is significantly thinner due to a 30-60% reduction in the density of synapses and apical and basal spines in old monkeys, indicating age-related degenerative changes of pyramidal cells.

Interestingly, the degree of cognitive decline associated with age progression or as a result of neurodegenerative conditions like Huntington's disease may be related in part to increases in KYNA thereby inducing a loss of dendritic spines. The role of KYNA in cognitive decline was initially suggested since KYNA antagonism of NMDA receptors could be overcome by not only serine, glycine or D-cycloserine, but also the cognitive enhancing drugs aniracetam and oxiracetam (Pittaluga *et al.*, 1997), suggesting these drugs may aim to increase NMDA function during cognitive decline. The significance for enhancing NMDA function becomes apparent since *in vivo* studies in rats show KYN and KYNA to increase with

age progression (Gramsbergen *et al.*, 1992; Wada *et al.*, 1994), while in human patients, KYNA and IDO is increased in the blood and brain with their levels correlating to the degree of cognitive decline (Pertovaara *et al.*, 2006). Interestingly, animal models where kynurenine has been injected to artificially elevate KYNA levels show a range of cognitive deficits when the animals were tested in a range of tasks (Alexander *et al.*, 2012; Pocivavsek *et al.*, 2012). Furthermore, knockout studies of the isoenzyme encoding for KAT II, which is responsible for the transamidation of KYN to KYNA, in a rat model showed a highly significant reduction in central levels of KYNA accompanied with improved cognitive scores in a range of behavioural testing paradigms including exploration, object recognition and passive avoidance learning tests (Potter *et al.*, 2010). These changes were confirmed to be due to reductions in KYNA since no change was seen in the levels of QUIN (Sapko *et al.*, 2006). Together, these studies suggest the gradual increase in KYNA with age progression may serve to increase NMDA receptor blockade causing a generalised disruption of synaptic transmission within the CNS. Since Ro61-8048 reduces spine density, presumably due to the increase in KYNA and its effects upon NMDA receptors, this suggests a potential mechanism whereby the continual increase in KYNA may contribute towards normal age-related cognitive decline. This process may be accelerated in neurodegenerative conditions such as Huntington's disease where advanced stages show significantly higher levels of KYNA. It is possible this potentiated increase may partly be responsible for the enhanced cognitive deficits observed in this fatal disorder. In support of this, it is interesting to note that Huntington's disease is also characterised by a significant reduction in dendritic spine density. A loss of spines is also reported in Alzheimer's disease (Ferrer *et al.*, 1990) from dentate granule cells (Einstein *et al.*, 1994) and neocortical and hippocampal pyramidal neurons (Fiala, *et al.*, 2002), and in Parkinson's disease where striatal neurons normally receiving dopaminergic projections (McNeill *et al.*, 1988) and neurons of the locus ceruleus and substantia nigra (Patt and Gerhard, 1993) display fewer dendritic spines. Since alterations in kynurenine metabolites are prominent during these disorders contributing towards the severity of the disease, the kynurenine pathway represents a common factor that may contribute towards cognitive decline by reducing spine density.

Interestingly, since schizophrenia is accompanied by a range of cognitive deficits with impairments in learning abilities, analysis of dendritic spines in schizophrenia subjects also reveal a significant reduction of spine density (Bennett, 2011) in pyramidal cells of the temporal and frontal cortex, while smaller spines have been reported in the striatum (Roberts *et al.*, 1996). However, the finding that chronic exposure to haloperidol in rats reduces spine density (Kelley *et al.*, 1997) confounds the reports obtained from schizophrenia subjects due to

the use of anti-psychotics during the disease. Although in a study with controls for the effects of anti-psychotic medication, spine loss was still evident in the dorsolateral prefrontal cortex (Glantz and Lewis, 2000), suggesting that schizophrenia itself including anti-psychotic treatment are both associated with a reduction in spine density. Since schizophrenia is accompanied by a range of cognitive deficits, and a reduced spine density will likely contribute to the emergence of these deficits, this highlights that although anti-psychotic treatment is efficacious in treating some of the symptoms for schizophrenia, their long-term use is likely precipitating deficits in neuronal morphology and brain volume previously attributed to the disease alone (Lieberman *et al.*, 2005; Moncrieff and Leo, 2010; Cacabelos *et al.*, 2011). Such observations warrant further research into new drugs that may correct for the abnormalities in spine deficits as oppose to contributing towards them. Furthermore, since environmental risk factors for schizophrenia such as maternal stress, prenatal infection and malnutrition (Fiala *et al.*, 2002; Benitez-Bribiesca *et al.*, 1999) are also associated with the permanent loss of dendritic spines, and the kynurenine pathway may be the linking convergent pathway, this further suggests that a kynurenine based therapy may be of benefit in reversing some of these deficits.

Since oxidative stress is involved in schizophrenia and may contribute towards the disease progression of a range of neurodegenerative diseases (Gilgun-Sherki *et al.*, 2001), it is interesting to note that reactive oxygen species also induce spine pathology. Pyramidal neurons in the CA1 hippocampus of rats display significantly reduced densities of dendritic spines 1 day after oxidative stress was induced by a 4 h exposure to ozone (Avila-Costa *et al.*, 1999). Also, a similar spine loss is seen in the prefrontal cortex, striatum and olfactory bulb, with vacuolation of dendrites and spines (Avila-Costa *et al.*, 2001), suggesting the loss of spines may be related to the levels of oxidative stress. This is of interest since a parallel study showed the protein expression of NFkB to be significantly increased in P21 animals when treated with Ro61-8048, implying that this may also contribute towards reduced spine density. Interestingly, this may be involved in mediating spine loss during maternal infection, since the reduced spine density following maternal infection may be related to the increase in markers indicative of oxidative stress together with reduced levels of glutathione at various postnatal ages ranging from P9-P510 (Paintlia *et al.*, 2008). Consistently, activated protein C, an anti-coagulant with anti-oxidant, anti-inflammatory and anti-apoptotic actions, administered to pregnant dams with LPS reversed the increase in white matter apoptosis and myelin deficits in P7 offspring (Yesilirmak *et al.*, 2007), while N-acetylcysteine, an anti-oxidant and NFkB inhibitor, administered 4 h post LPS treatment in pregnant rats prevented deficits in LTP and spatial learning (Lante *et al.*, 2008). This further emphasises the potential impact of oxidative



stress on cognitive deficits and spine loss, and suggests the kynurenine pathway may be involved in mediating a reduction in spine density following maternal immune activation.

### 1.5 General Conclusion and Significance of Study

The general theme of this project was to examine the role of the kynurenine pathway on brain development, to understand whether manipulations in this pathway during development could enhance the subsequent onset of schizophrenia during adulthood. This theory was based upon findings showing environmental factors including stress, malnutrition and infection could adversely affect the pregnant mother and foetus thereby increasing the risk for neurodevelopmental disorders later in life (Meyer *et al.*, 2008d; Meyer and Feldon, 2009, 2012, Meyer, 2013). Maternal infection can produce a variety of cognitive and behavioural deficits in the offspring during adulthood, resembling those seen in schizophrenia (Brown *et al.*, 2009), however, the molecular basis for these behavioural abnormalities remain poorly understood. It is believed bacterial or viral infectious agents activate the maternal immune response to increase the concentration of pro-inflammatory cytokines which is believed to mediate developmental damage. Indeed, pro-inflammatory cytokines including IL-1 $\beta$ , TNF- $\alpha$  and IL-6 amongst others, can impair cellular differentiation and negatively regulate the survival of neurons within the CNS (Ling *et al.*, 1998; Potter *et al.*, 1999; Jarskog *et al.*, 1997). A pro-inflammatory cytokine response produces a variety of noxious metabolites thereby increasing the levels of oxidative stress which can also regulate cell survival during development. Interestingly, some of the most convincing data to emerge from the cytokine literature are reports demonstrating the prenatal administration of rodents with the pro-inflammatory cytokine IL-6, is capable of recreating the cognitive and behavioural deficits resembling those associated with poly(I:C) or LPS treatment (Smith *et al.*, 2007). While there is convincing evidence implicating a role for cytokines in mediating developmental damage, since the immune activation as evidenced by an increase in pro-inflammatory cytokines are a relatively transient phenomenon, rarely persisting beyond 24 h, this fails to explain the long-term changes in brain and behaviour precipitated by the initial insult. It is therefore likely that activation of the maternal immune response triggers a variety of signalling changes that may continue once the cytokine profile returns to normal. Furthermore, although reports suggesting novel anti-inflammatory treatment to prevent the effects of maternal infection, anti-inflammatory agents administered to the pregnant mother is also associated with the rise in the risk of schizophrenia (Sorensen *et al.*, 2004). Therefore, novel treatments for such conditions will likely arise from the identification of biochemical pathways that are activated

and dis-regulated during maternal infection that may contribute to developmental damage. One such biochemical pathway that may mediate this link between immune activation and the subsequent psychopathology may be the kynurenine pathway since this is activated by pro-inflammatory cytokines, and is involved in a range of affective and neurodegenerative diseases. Indeed, infectious agents inducing IDO activation can result in persistent changes in kynurenine levels for substantial periods, and may be involved in mediating part of the developmental damage induced by pro-inflammatory cytokines (Alberati-Giani and Cesura, 1998). The persistent increase in the neurotoxic kynurenine metabolite QUIN during infection can induce excitotoxic damage via the activation of the NMDA receptor, production of reactive oxygen species and by inducing mitochondrial dysfunction (Ma *et al.*, 1997; Bordelon *et al.*, 1997; Santamaría *et al.*, 2003), effects antagonised by its antagonist, KYNA. These principal mechanisms mediate much damage in neurodegenerative conditions such as Alzheimer's, Parkinson's and Huntington's disease. Therefore, since the kynurenine pathway can be activated by a range of environmental factors, including infection and stress, it is a plausible hypothesis that the effects of maternal immune activation on mediating the subsequent risk in schizophrenia may be mediated in part by the activation and dysfunction of the kynurenine pathway. This implies that modifying the activity of the kynurenine pathway during development, in the absence of maternal immune activation, may also precipitate developmental damage that may be of relevance to schizophrenia. Since this pathway may provide a convergent mechanism by which environmental factors such as stress or infection may enhance the susceptibility to schizophrenia or related disorders, this further suggests that drugs capable of correcting kynurenine concentrations during development may be of benefit for the novel treatment of developmental disorders.

### 1.5.1 Main Findings – Prenatal Treatment With poly(I:C)

In the poly(I:C) model of maternal immune activation, we have reported molecular changes occurring in P21 animals. While some changes including a reduced GluN1 expression is generally recognised as major contributors towards the pathophysiology of schizophrenia, interestingly, we have shown maternal infection is not associated with changes in protein markers important in neuronal development and axonal guidance, suggesting that the overall cortical neuronal connections may largely be unaffected. The range of protein changes during maternal infection provides a mechanism by which environmental factors such as stress and infection may increase the susceptibility of schizophrenia. A major finding is the substantial reduction in  $\alpha$ -synuclein following poly(I:C) treatment at P21 suggesting, that although Parkinson's disease is considered a neurodegenerative as opposed to a neurodevelopmental disorder, it is plausible that developmental damage may predispose



individuals to a heightened risk of developing Parkinson's disease following subsequent exposure to environmental toxins (Tien *et al.*, 2013; Bobyn *et al.*, 2012). Therefore, although maternal infection increases the risk for schizophrenia, in light of the substantial loss of  $\alpha$ -synuclein, it is proposed that maternal infection may also predispose individuals to a heightened susceptibility for developing Parkinson's disease during adulthood.

### *1.5.2 Main Findings – Prenatal Treatment With Ro61-8048*

To examine the effects of the kynurenine pathway on brain development, we sought to assess the potential consequences of disrupting kynurenine metabolites prenatally on the normal physiological activity of the kynurenine pathway and its subsequent role in developmental disorders. Therefore, Ro61-8048 was used to inhibit KMO thereby increasing the transamidation of KYN to KYNA, since studies suggest cell polarity and development is largely controlled by the gradient of signal antagonists as opposed to an agonist. Administration of Ro61-8048 during pregnancy has shown to produce a variety of protein, electrophysiological and morphological changes in rodents during adulthood, emphasising the great importance of this pathway on brain development. Indeed, our results suggest the kynurenine pathway is constitutively active during foetal development and is intimately involved in normal brain development. This marks a minor breakthrough within the field of kynurenine research since its role has not been assessed during development. While KMO inhibitors are investigated as potentially novel therapeutics for the treatment of Parkinson's, Alzheimer's and Huntington's disease, if such therapy were to successfully complete clinical trials, the data presented in this thesis strongly suggest that the use of KMO inhibitors could prove deleterious in pregnant women suffering from such diseases. Furthermore, the significance of this research is not only restricted to the actual inhibition of KMO, or any other enzyme in the pathway, since these compounds were used experimentally to induce alterations in kynurenine metabolites only, to reflect changes that may be caused due to environment factors. Indeed, since infection and stress can induce activation of the kynurenine pathway, the results presented in this thesis lend support to the theory that the kynurenine pathway may be a convergent mechanism by which environmental factors such as infection and stress induce developmental abnormalities in the offspring during adulthood. This also implies that a novel kynurenine based therapy may be of major benefit in treating such conditions circumventing the detrimental effects of employing agents to block inflammatory agents or to increase anti-inflammatory cytokines. Further research into the developmental role of the kynurenine pathway is warranted considering current anti-psychotics may be responsible for reductions in cortical and hippocampal volume (Lieberman *et al.*, 2005; Moncrieff and Leo, 2010; Cacabelos *et al.*, 2011), implying significant toxicity of

these drugs. Also, since the widespread use of NMDA antagonists are associated with significant side effects, due to the non-specific inhibition of all its receptors, contributing primarily to the principal reason for their failures in clinical trials, the kynurenine pathway represents an attractive drug target in localising effects to specific regions. This is achieved since basal levels of kynurenines are usually low and principally change in localised regions affected by damage only. This may be a predominant reason underlying the increased patenting of compounds related to these disorders (Muchowski *et al.*, 2011; Zisapel *et al.*, 2012; Andersen *et al.*, 2012).

The results presented within this thesis strongly suggest that disruption of the kynurenine pathway during development may be related to the onset of schizophrenia later in life. The results demonstrate changes in kynurenines prenatally may alter the function of the NMDA receptor permanently, since Ro61-8048 induced significant impairments in the protein expression of the NMDA receptor subunits GluN2A and GluN2B in embryo brains as early as 5 h post-treatment, while persisting into P60 young adult rats. Interestingly, while Ro61-8048 induces significant impairments in the NMDA receptor, it does not alter the immediate expression profiles of protein markers related to neuronal development and axonal guidance. Interestingly, Ro61-8048 precipitates delayed effects in the expression of these proteins primarily becoming evident at P60, including substantial changes in the netrin family of proteins. Since these proteins are intimately involved in axonal guidance, their altered expression profiles may likely induce significant impairments in the neuronal connections made during adulthood following neurogenesis in the hippocampus and cerebellum in the adult brain.

Perhaps one of the most convincing finding linking the altered activity of the kynurenine pathway with schizophrenia, is the significant change in the protein expression of DISC-1 in the cortex. DISC-1 is heavily linked with the emergence of schizophrenia and is identified as a schizophrenia susceptibility gene. Also, cognitive deficits following increases in KYNA may be related to its propensity to substantially elevate the levels of excitatory glutamate transporters that may be involved in enhancing the rate of glutamate activity termination in the hippocampus, since studies of altered glutamate signalling in the hippocampus are associated with cognitive deficits. This increase in glutamate transporters was also a highly selective change, since Ro61-8048 treatment did not affect the density of GABA transporters. Furthermore, considering the major deficits in cognition during schizophrenia, that are accompanied by a reduction in spine density, it is an interesting parallel with the present study reporting a significant reduction in dendritic spine density in the P60

hippocampus following prenatal treatment with Ro61-8048. A reduction in spines were observed in both apical and basal dendrites and may be of relevance to schizophrenia, but also to other neurodegenerative conditions like Parkinson's and Alzheimer's disease that are also characterised by reductions in spine density. Considering the principal parameters affected by Ro61-8048 treatment, including the NMDA receptor, glutamate transporters and spine density, this suggests that the kynurenine pathway may principally disrupt cognitive abilities during schizophrenia.

## 1.6 Limitations of the Present Study and Future Work

One of the major limitations of the present study is that although a range of changes have been noted at various time points, it failed to establish a mechanism underlying these changes, resulting in the discussion of these protein alterations being largely descriptive in nature. Therefore, future work should have a greater focus on the mechanism that may underlie these neurochemical alterations, and experiments on neuronal cell cultures would provide the best means to do that. Indeed, cell cultured experiments should also examine the relative role of some of these proteins changes. For example, the deletion of doublecortin or DISC-1 and the effects it has upon cerebellar and cortical neurons should be examined to see in what way their development or function may be affected. To examine the interaction of poly(I:C) on kynurenines, cell cultures using both embryonic and adult neuronal cell lines should be used to investigate the consequence of immune activation in the absence of IDO or QUIN, and to see whether it is associated with a reduction in neurotoxicity. The effects of Ro61-8048 should also be examined upon immune stimulation in cultured experiments to see whether it offers any protection against infection. Since Ro61-8048 appears to mainly produce delayed effects, it would be useful to pre-treat neuronal cultures before applying poly(I:C). The effects of Ro61-8048 in inducing developmental damage should be compared with another model where KYNA is increased via an alternative mechanism. Either kynurenine could be administered with probenecid to increase central KYNA during development. The principal reason for this is to verify that the changes reported in this study are only attributed to changes in kynurenines during development and not due to other actions of the drug. Although Ro61-8048 is reported to be a selective inhibitor of KMO, whether there exist other mechanisms of action, this remains unreported and therefore unknown.

Since some of the protein markers examined vary considerably during development and adulthood, and while every effort was made to optimise the antibody dilutions to either

substantially reduce its dilution for proteins such as doublecortin, RhoA, RhoB and DCC for the embryo work since these proteins are present at much higher levels, or increase the antibody dilution for doublecortin and DCC during the P60 experiments, occasionally the western blotting still produced a strong signal, sometimes appearing overexposed. In the case of synaptic protein markers including VAMP-1, synaptophysin and synaptotagmin, the lack of changes following poly(I:C) and Ro61-8048 administration may be related to the fact that since these proteins are expressed in very high amounts, subtle changes in their protein expression may be out-with the detection limit for the western blotting technique, but they may also be related to the fact that these blots, usually producing very strong signals, may have been overexposed thereby compromising their integrity in demonstrating a clear change. This is especially important for synaptophysin, since loss of GluN1 and poly(I:C) administration is associated with a reduction in synaptophysin, while in the presence of both these factors, our experiments revealed no change. For this reason, the assessment of synaptic proteins may have been better suited for examination by a quantitative method using real-time polymerase chain reaction.

Another major limitation of the experiments is that for P21 animals, protein expression was examined in the entire halves of the cerebral hemisphere as opposed to regions like the P60 experiments. Therefore, discrete changes occurring in some brain regions may have been masked, or that changes occurring in different regions and in opposite directions, as often noted in other studies, would produce no overall net change in our half brain lysates. Therefore, for these experiments a more accurate conclusion of the results would be that there was no overall net change in protein expression when examined in the half brains, while subtle more localised changes may exist. This limitation may also apply to the P60 midbrain regions since this region is of interest to a range of disease including schizophrenia, Parkinson's and Huntington's disease, and most studies have reported neurochemical alteration within this region to be changing in highly localised regions of the substantia nigra or in the nucleus accumbens, often in opposite directions. Since this would reduce the ability for a real change to be detected in the western blotting technique, the subcellular examination of critical markers including tyrosine hydroxylase and 5HT-2<sub>C</sub>, may be better suited for assessment with immunocytochemistry.

Since the data from the present study implies alterations in kynurenines during development may be involved in schizophrenia, P60 animals should be tested in a range of cognitive, learning and behavioural paradigms including examination of prepulse inhibition, latent inhibition, enhanced sensitivity to NMDA channel blockers or amphetamine and

anxiety. Furthermore, depending upon the deficits, the therapeutic efficacy of anti-psychotic medication should be assessed to examine whether the animals are responsive to treatment. Some of the most exciting work remains in comparing the effects of disrupting the kynurenine pathway in genetic models of schizophrenia. This would be useful in identifying interactions between genetic and kynurenine-based environmental factors in an attempt to identify how these factors influence vulnerability towards schizophrenia. These experiments could involve Ro61-8048 treatment in wild-type animals which is then compared with the effects of Ro61-8048 treatment in animals genetically modified to exhibit one or many schizophrenia susceptibility genes. Some of the major susceptibility genes worth examining in this model include neureglin-1 (NRG-1), catechol-O-methyltransferase (COMT) and disrupted in schizophrenia-1 (DISC-1).

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